REMARKS

The presently claimed invention features methods for identifying candidate therapeutic agents for the treatment of prostate cancer. The claimed methods entail screening test compounds to identify those compounds that reduce the expression of alpha-methylacyl CoA racemase mRNA.

Objections to the claims

The Examiner objected to claim 33 as including non-elected species. Applicants have amended claim 33 to refer to SEQ ID NO:3, an elected species.

The Examiner objected to claim 34 as not setting forth the "metes and bounds" of the invention with respect to the term "stringent conditions." Applicants have amended claim 34 to specify that the nucleic acid molecule "selectively" hybridizes to the alpha-methylacyl CoA racemase mRNA under specified hybridization and washing conditions. Support for this amendment is found in the specification at page 83.

Rejections Under 35 U.S.C. §112, first paragraph

The Examiner rejected claims 33 and 34 under 35 U.S.C. §112, first paragraph as allegedly not enabled. Applicants respectfully traverse this rejection.

The Examiner argued that the specification does not enable one skilled in the art to use the claimed methods to identify candidate therapeutic agents for the treatment of prostate cancer. The Examiner provided four arguments for finding that the claims are not enabled: 1) there is no evidence that alpha-methylacyl-CoA racemase ("racemase") is "responsible" for prostate cancer development; 2) *in vitro* cultured prostate cancer lines cannot be used to predict results in prostate cancer *in vivo*; 3) anti-cancer drug discovery is "highly unpredictable"; and 4) gene therapy and *in vivo* antisense therapy are "unpredictable". As explained in greater detail below, Applicants disagree with the Examiner's conclusions based on the arguments set forth.

Before addressing each of the Examiner's four arguments, Applicants first address the overall context of the Examiner's rejection of the claims as allegedly not enabled. Many of the Examiner's reasons for concluding that the claims are not enabled appear to based on skepticism regarding whether a compound that inhibits racemase expression is a candidate therapeutic agent for treating prostate cancer. However, the enablement standard does not require that it be conclusively demonstrated that the claimed methods are capable of identifying a successful prostate cancer therapy. Rather, the specification must teach one skilled in the art how to use the claimed methods to identify inhibitors of racemase expression that are candidate therapeutic agents. The claimed methods are enabled and useful if they allow one skilled in the art screen a large number of compounds in order to identify a subset that have potential utility as therapeutic agents and thus merit the expense and effort of additional testing to determine if any are indeed useful therapeutic compounds for the treatment of prostate cancer.

The claims are enabled irrespective of whether racemase is "responsible' for prostate cancer

The Examiner argued that the claims are not enabled because "there is no indication that SEQ ID NO:1 or 3 is responsible for prostate cancer." It is Applicants' position that the claims are enabled irrespective of whether racemase is "responsible" for prostate cancer in the sense of being the cause of prostate cancer. Moreover, there is considerable evidence that increased expression of racemase plays a role in the development and/or progression of prostate cancer.

First, as described in the present specification, Applicants found that racemase mRNA and protein is expressed at a higher level in actual clinical prostate tumor sample and prostate tumor metastases than in clinical samples of normal prostate tissue. Numerous studies on large patient populations have confirmed this result (see, e.g., Beach et al. 2002 *Am. J. Surgical Pathol.* 26:1588 (Exhibit A); Kuefer et al. 2002 *Am. J. of Pathol.* 161:841 (Exhibit B); Luo et al. 2002 *Cancer Res.* 62:2220 (Exhibit C); Rubin et al. 2002 *JAMA* 287:1662 (Exhibit D); and Jiang et al. 2001 *Am. J. Surgical Pathol.* 25:1397 (Exhibit E); copies enclosed).

Second, as Luo et al. (*Cancer Res.* 62:2220, 2002; Exhibit C) explains, certain aspects of the biological activity of racemase may have particular relevance for prostate cancer. Racemase catalyzes the conversion of (R)-1-methyl branched-chain fatty acyl-CoA esters to (S)-stereoisomers, which, unlike the (R)-stereoisomers, can serve as substrates for branched-chain acyl-CoA oxidase during peroxisomal oxidation. Luo et al. explains that two aspects of this oxidation pathway may have particular relevance for prostate carcinogenesis: (a) the main sources of branched-chain fatty acids in humans have been implicated as dietary risk factors for prostate cancer; and (b) peroxisomal β-oxidation generates hydrogen peroxide, a potential source of pro-carcinogenic oxidative damage (Luo at page 2220, citations omitted)." Thus, Luo confirms biological activity of racemase provides a scientifically credible basis supporting the proposition that racemase plays a role in the development and/or progression of prostate cancer.

Third, a recent study concluded that sequence variants in the racemase gene are associated with prostate cancer risk (Zheng et al. 2002 *Cancer Res.* 62:6485; Exhibit E). This study examined 17 sequence variants, including 10 missense mutations in 159 HPC families, 245 sporadic prostate cancer cases an 222 non-prostate cancer controls. The fact that certain racemase sequence variants are associated prostate cancer risk further supports the proposition that racemase plays a role in the development and/or progression of prostate cancer.

Moreover, even if racemase is not *per se* responsible for causing prostate cancer, racemase expression is indicative of prostate cancer and prostate cancer metastasis. Therefore, the presently claimed methods are enabled for the identification of candidate therapeutic agents for treatment of prostate cancer irrespective of whether racemase "causes" prostate cancer.

It is evident that there is a sound scientific basis for concluding that inhibitors of racemase expression could be useful as therapeutic agents for the treatment of prostate cancer. This conclusion is not dependent on determining that increased racemase expression is "responsible" for prostate cancer, as the examiner appears to suggest.

Cultured cells can be used as a model of prostate cancer

The Examiner cited a number of publications describing differences between cultured cancer cell lines and clinical cancer cell samples. None of these publications discuss racemase expression in prostate cancer cell lines.

In contrast, studies have shown that racemase is expressed at a higher level in cell lines derived from metastatic prostate cancer than benign cell line. For example, Kuefer et al. (*Am J. Pathology* 161:841, 2002; Exhibit B) found that racemase mRNA was expressed at high level in LNCaP cells, a hormone responsive cell line derived from a metastatic prostate cancer; at a moderate level in PC3 and DU145 cells, hormone-independent cell derived from metastatic prostate cancer; and was nearly absent in RWPE-1 cells, a benign prostate cell line. Thus, various prostate cell lines do in fact resemble clinical prostate samples with respect to racemase expression.

While there can be differences between cultured cancer cell lines and clinical cancer cell samples, cell lines are commonly used to study cancer because they are useful despite the presence of some differences. In the present instance, one skilled in the art would expect that in vitro assays would be useful for identifying compounds that inhibit racemase mRNA expression according to the presently claimed methods. Thus, it is improper for the Examiner to simply dismiss cancer cell lines as worthless for drug screening.

The claims are enabled despite the challenges of anticancer drug discovery

The Examiner argued that the claims are not enabled because "the art of anticancer drug discovery is unpredictable". According to the Examiner, because of this unpredictability, "in the absence of experimental evidence, no one skilled in the art would accept the assertion that the screened compounds that inhibit the mRNA expression of SEQ ID NO:1 or 3 in vitro would be useful for treating prostate cancer in a patient as claimed." The Examiner's argument ignores the language of the claims and fails to consider the reality of drug discovery.

First, the Examiner's argument improperly ignores the language of the claims. The claims are directed at the identification "of candidate therapeutic agents for the treatment of prostate cancer" Thus, the proper standard for evaluating the enablement and usefulness of the claimed methods is not whether an inhibitor of racemase mRNA expression identified using the claimed method is certain to be useful for treatment of prostate cancer. Any candidate therapeutic agents identified in an in vitro screen will, of course, require considerable further study and testing using other assays and ultimately clinical trials before it can be determined whether it is useful for treating prostate cancer in a patient. This is not a reason to find that in vitro screening claims are not enabled. The value of in vitro screening using a specific target, e.g., racemase, is based on the ability of such screening to narrow the group of compounds that are worthy of such further study.

Second, the Examiner's argument simply ignores the reality of target-based drug discovery. The Examiner, citing Gura (*Science* 278:1041, 1997), makes much of the fact that "many thousands of drugs have shown activity in ether cell or animal models but ... only 39 have actually been shown to be useful for chemotherapy". However, this is hardly an argument for finding that the present claims are not enabled. Applicants respectfully point out the presently claimed methods are but one aspect in the whole of the drug discovery process. Despite the fact that few compounds identified in screens ultimately prove to be a successful therapy, for various reasons, large pharmaceutical companies, small start-up companies, and government and academic researchers the world over spend countless hours and many, many millions of dollars using in vitro target-based screens to identify candidate therapeutic agents for the treatment of cancer. Researchers are well aware of the various challenges of the drug discovery process noted by the Examiner. However, they continue to use target-based screening because they believe that such screens are useful for identifying candidate therapeutic agents. The Examiner cannot simply dismiss a scientific approach that is so widely used simply because the ultimate goal of the whole of the therapeutic discovery process is difficult to achieve.

The claims are enabled irrespective of the unpredictability of gene therapy or antisense therapy

The Examiner argued that the claims are not enabled because gene therapy and in vivo antisense therapy are "unpredictable". The Examiner provides a detailed discussion of some of the difficulties that can occur in gene therapy and antisense therapy. However, the Examiner's concerns are misplaced and undue. Assuming, without conceding that gene therapy and antisense therapy are not useful for treating prostate cancer and that nucleic acids are not useful as test compounds in the claimed screening methods, there remain many, many compounds, e.g., small molecules that can be screened and may prove to be useful therapeutic agents.. Thus, reconsideration and withdrawal of the rejection is requested.

"Stringent hybridization conditions"

The Examiner rejected claim 34 as allegedly not enabled because, according to the Examiner, "it would be expected that a substantial number of the hybridizing molecules encompassed by the claims would not share either structural or functional properties with polynucleotides with the polynucleotides of SEQ ID NO:1 or 3."

Applicants have amended claim 34 to include specific hybridization conditions and to specify that the molecules "selectively" hybridize.

CONCLUSION

In view of the forgoing, Applicants respectfully request that the rejections under 35 U.S.C. §112, first paragraph be withdrawn.

Enclosed is a Petition for Extension of Time with the appropriate fee. Please apply any other charges or credits to deposit account 06-1050.

Respectfully submitted,

Date: 18 Sept 2003

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Exhibit A

The American Journal of Surgical Pathology 26(12): 1588–1596, 2002

P504S Immunohistochemical Detection in 405 Prostatic Specimens Including 376 18-Gauge Needle Biopsies

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P504S is a recently described, prostate cancer-specific gene that encodes a protein involved in the beta-oxidation of branched chain fatty acids. A recent study has shown that immunohistochemical detection of P504S gene product is a sensitive and specific marker of prostatic carcinoma in formalin-fixed, paraffin-embedded tissues. We performed a detailed analysis of P504S protein expression in a large series of prostate and bladder specimens with special emphasis on staining in specific morphologic patterns of prostatic adenocarcinoma, posthormonal and radiation therapy cases, and invasive urothelial carcinoma. A total of 366 prostate needle core biopsies from 124 patients with prostate cancer, 10 biopsies from 2 patients without prostate cancer, 28 prostatectomy specimens (16 with specific morphologic patterns, 7 posthormonal therapy and 5 postradiation therapy specimens), 5 bladder specimens with invasive urothelial carcinoma, and a single transurethral resection specimen from a patient with hormonally treated prostate cancer and invasive urothelial carcinoma were stained with P504S monoclonal antibody at a 1:250 dilution using standard heat-induced epitope retrieval and avidin-biotin technique. Extent (0, no staining; 1+, 1–10% staining; 2+, 11–50% staining; 3+, ≥51% staining) and location (luminal, subluminal, and diffuse cytoplasmic) of immunoreactivity in carcinoma and benign tissues were recorded. A total of 153 of 186 biopsies (82%) with prostatic adenocarcinoma stained for P504S. Pseudohyperplastic, atrophic, ductal, and mucinous prostatic carcinomas stained similarly, as did cases treated with hormone or radiotherapy. In 81 of 377 (21%) foci of benign prostatic tissue there was staining that was almost always focal, faint, and noncircumferential. Seminal vesicles did not stain for P504S. Five of six (83%) specimens with invasive urothelial carcinoma had 2+ staining and one case had focal staining. We conclude that immunohistochemistry for P504S has potential utility in the diagnosis of prostate cancer, including those treated by hormones and radiation. Circumferential luminal to subluminal and diffuse cytoplasmic staining is the most specific staining pattern for prostatic carcinoma and is almost never associated with benign prostatic tissue. However, a negative P504S immunostain does not automatically rule out prostate cancer, as 18% of cases were negative. Additionally, occasional benign glands, high-grade prostatic intraepithelial neoplasia, atypical adenomatous hyperplasia, and urothelial carcinoma may express P504S. Therefore, we think that P504S is best used only in conjunction with strict light microscopic correlation and preferably with high molecular weight cytokeratin immunostaining.

Key Words: Prostate carcinoma—Urothelial carcinoma—Immunohistochemistry—Cytokeratins—*P504S* gene—*α-Methylacyl-CoA Racemase* gene—*AMACR* gene.

Am J Surg Pathol 26(12): 1588–1596, 2002.

P504S, a prostate cancer specific gene, was recently discovered by using cDNA library subtraction with subsequent high throughput microarray screening of human prostatic tissues.[12] Other groups have confirmed this finding independently.[9] *P504S*, also referred to as the *AMACR* gene, encodes α-methylacyl-CoA racemase, a 382-amino acid protein involved in the beta-oxidation of branched-chain fatty acids.[9] Immunohistochemistry using a rabbit monoclonal antibody to P504S gene product (henceforth referred to as P504S) showed expression in all cases of prostate carcinoma studied from prostatectomies, needle biopsies, and transurethral resection of the prostate (TURP) specimens.[6] Jiang et al. also showed P504S expression in high-grade prostatic intraepithelial neoplasia (HGPIN), but not in benign glands, which showed only focal and weak staining, or in small benign glandular proliferations (including atrophy), which were negative.[6]

We undertook a detailed morphologic and immunohistochemical correlative study of P504S expression in a large series of prostatic needle biopsy specimens showing a wide range of benign and malignant prostatic glan-

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dular proliferations to validate the findings from the Jiang et al. study.[6] Further, we paid particular attention to patterns of staining in benign and malignant epithelia and to the immunoexpression in distinctive small acinar proliferations and seminal vesicles. Additionally, we evaluated P504S expression in different grades of prostate carcinoma, unusual morphologic patterns of conventional prostatic carcinoma, variants of prostatic carcinoma, prostatic carcinomas treated with hormonal and radiotherapy, and in invasive urothelial carcinomas.

MATERIALS AND METHODS

Prospectively collected intermediate levels from a total of 376 prostate needle core biopsies (344 consecutive biopsies) from 126 patients and 3 bladder biopsies, 2 cystectomy specimens, and a single TURP specimen were obtained from the Emory University Hospital (n = 350) and Henry Ford Hospital (n = 32) surgical pathology files (Table 1). The 28 prostatectomy specimens were collected from Emory University Hospital (unusual morphologic patterns and variants), Henry Ford Hospital (posthormonal treatment cases), and Wayne State University (postradiation treatment salvage prostatectomies). A total of 186 of the prostate biopsies from 111 patients had variable amounts of prostatic carcinoma in the biopsy. A total of 180 of the prostate biopsies were benign, and these were collected from two patients without prostatic carcinoma (10 biopsies) and 79 patients with prostatic carcinoma in other biopsies (170 biopsies). Thus, several patients had both benign biopsies and biopsies with prostatic carcinoma that were included in this study. Twelve of the prostatectomy specimens had posttherapy prostatic adenocarcinoma, including seven posthormonal and five postradiation specimens. Eight of the prostatectomy specimens had unusual morphologic patterns of conventional prostatic carcinoma, including pseudohyperplastic (n = 6) and atrophic (n = 2) patterns, and eight had variants of prostatic carcinoma including ductal (n = 5) and mucinous (n = 3). The three bladder biopsies and two cystectomy specimens had invasive urothelial carcinoma. The single TURP specimen had hormonally treated prostate carcinoma and invasive urothelial carcinoma.

Tissue Processing

All tissue was fixed in 10% neutral buffered formalin and processed routinely. In all prostate needle biopsy cases, the formalin-fixed, paraffin-embedded tissue blocks were cut at five levels into 5-μm sections and transferred to charged glass slides. Levels 1, 3, and 5 were stained with hematoxylin and eosin for routine light microscopic diagnosis. The study was performed retrospectively on prospectively saved levels 2 or 4 from each specimen.

Immunohistochemistry

For epitope retrieval before immunostaining, slides were pretreated with 0.01 mol/L citrate buffer, pH 6, for 8 minutes in a pressure cooker. The slides were incubated with a rabbit monoclonal antibody (Mab) to P504S (clone 13H4) diluted at 1:250, which was obtained from Corixa Corporation (Seattle, WA, USA). The DAKO Envision Plus detection system was then used for antibody localization, following the vendor's protocol. Sections were then washed, developed with

TABLE 1. *Specimens included in study*

Needle core prostate biopsies	No. of biopsies	No. of patients
Cases with prostate CA in at least one biopsy		
Benign biopsies	180	79
CA biopsies	186	111
Cases without prostate CA	10	2
Total	376	126
Prostatectomies	**No. of specimens**	**No. of patients**
Variants of prostate CA	8	8
Unusual morphologic patterns of prostate CA	8	8
Post-hormonal treatment	7	7
Post-radiation treatment	5	5
Total	28	28
Cases with invasive urothelial CA	**No. of specimens**	**No. of patients**
Biopsies	3	3
Cystectomy	2	2
Total	5	5
TURP with urothelial and prostate CA	1	1

CA, carcinoma; TURP, transurethral resection of prostate.

3,3'-diaminobenzidine and hydrogen peroxide, and counterstained with hematoxylin.

Morphologic and Immunohistochemical Evaluation

The hematoxylin and eosin-stained and immunostained sections of all specimens were reviewed by three study pathologists (R.B., P.S., M.B.A.). In biopsies with carcinoma, a Gleason score was assessed for each biopsy. In biopsies without carcinoma, benign prostatic epithelium was classified as atrophy, transitional metaplasia, postatrophic hyperplasia, basal cell hyperplasia, and benign prostatic tissue not otherwise specified. Because an individual biopsy may have contained multiple different categories of benign prostatic tissue, each focus of benign prostatic tissue was independently evaluated on each biopsy. If a single benign pattern predominated, only that pattern was scored. Biopsies that were composed predominantly or entirely of carcinoma were not scored for benign prostatic tissue. Observations regarding P504S staining in HGPIN are not included in this article and are the subject of a separate study.

The extent of immunostaining was recorded as 0 (no positive cells), 1+ (1–10% positive cells), 2+ (11–50% positive cells), or 3+ (≥51% positive cells) in the prostate needle biopsies and bladder specimens. Circumferential or partial gland staining and the location of staining including luminal, subluminal, and cytoplasmic were also evaluated. Staining intensity was only recorded when it was particularly weak or strong in a given specimen, realizing that intensity interpretation is subjective, of limited practical value, and dependent on antibody dilution and laboratory variability.

RESULTS

Morphology

Prostatic carcinoma in needle biopsies had Gleason scores that ranged from 5 to 10. Eighty-four of 186 biopsies had Gleason scores of 5–6, 94 of 186 biopsies had Gleason scores of 7–8, and 8 of 186 biopsies had Gleason scores of 9–10. The morphology of the prostatic carcinoma variants, the unusual morphologic patterns of prostate cancer, and the posttherapy specimens was reconfirmed (Table 1).

P504S Expression in Prostate Carcinoma in 18-Gauge Needle Biopsies

Prostatic adenocarcinoma in 153 of 186 (82%) biopsies showed P504S expression, with at least 2+ positivity in 135 (72.6%) (Table 2; Fig. 1). P504S expression was seen in a finely granular, heterogeneous pattern (Fig. 1). The location of staining was usually luminal to subluminal and circumferential, but some specimens showed diffuse cytoplasmic staining (Fig. 1). Moderate staining intensity predominated, but weak and intense staining was also found. Gleason score appeared to have no correlation with the amount of P504S staining (Table 3).

TABLE 2. *P504S staining of prostatic aderocarcinoma in needle biopsies*

Amount of staining	No. of biopsies	Percentage
0	33	18
1+	18	10
2+	75	40
3+	60	32
Total	186	100

P504S Expression in Unusual Morphologic Patterns and Variants of Prostatic Carcinoma in Prostatectomies

Five of six (83%) specimens with pseudohyperplastic patterns, two of two (100%) specimens with atrophic patterns, five of five (100%) specimens with ductal adenocarcinoma, and two of three mucinous carcinomas (67%) were P504S positive (Figs. 2, 3).

P504S Expression in Prostate Carcinoma Treated With Hormonal or Radiation Therapy

Eight of eight specimens (including one TURP case) of hormonally treated carcinoma and four of five specimens of radiation-treated carcinoma were P504S positive. In both posthormonal and postradiation therapy specimens, the staining amount was inversely proportional to the degree of treatment effect (Fig. 4). Thus, foci of carcinoma with marked treatment effect had less staining than foci with minimal treatment effect in the same specimen.

P504S Expression in Benign Prostatic Epithelia in 18-Gauge Needle Biopsies

Benign glandular prostatic tissue was mostly negative. However, overall, 81 of 377 (21%) foci of benign prostatic tissue had some staining. Sixty-four of 270 (24%) biopsies with benign prostatic epithelium (benign not otherwise specified) had 1+, faint, and noncircumferential luminal staining (Fig. 5). In 14 of 270 (5%) biopsies with benign glands not otherwise specified, there was 2+ to 3+ staining that was also almost always weak, luminal, and noncircumferential. No staining was encountered in the specific small gland proliferations of postatrophic hyperplasia (n = 10), transitional metaplasia (n = 17), and basal cell hyperplasia (n = 1). Three of 79 (4%) foci

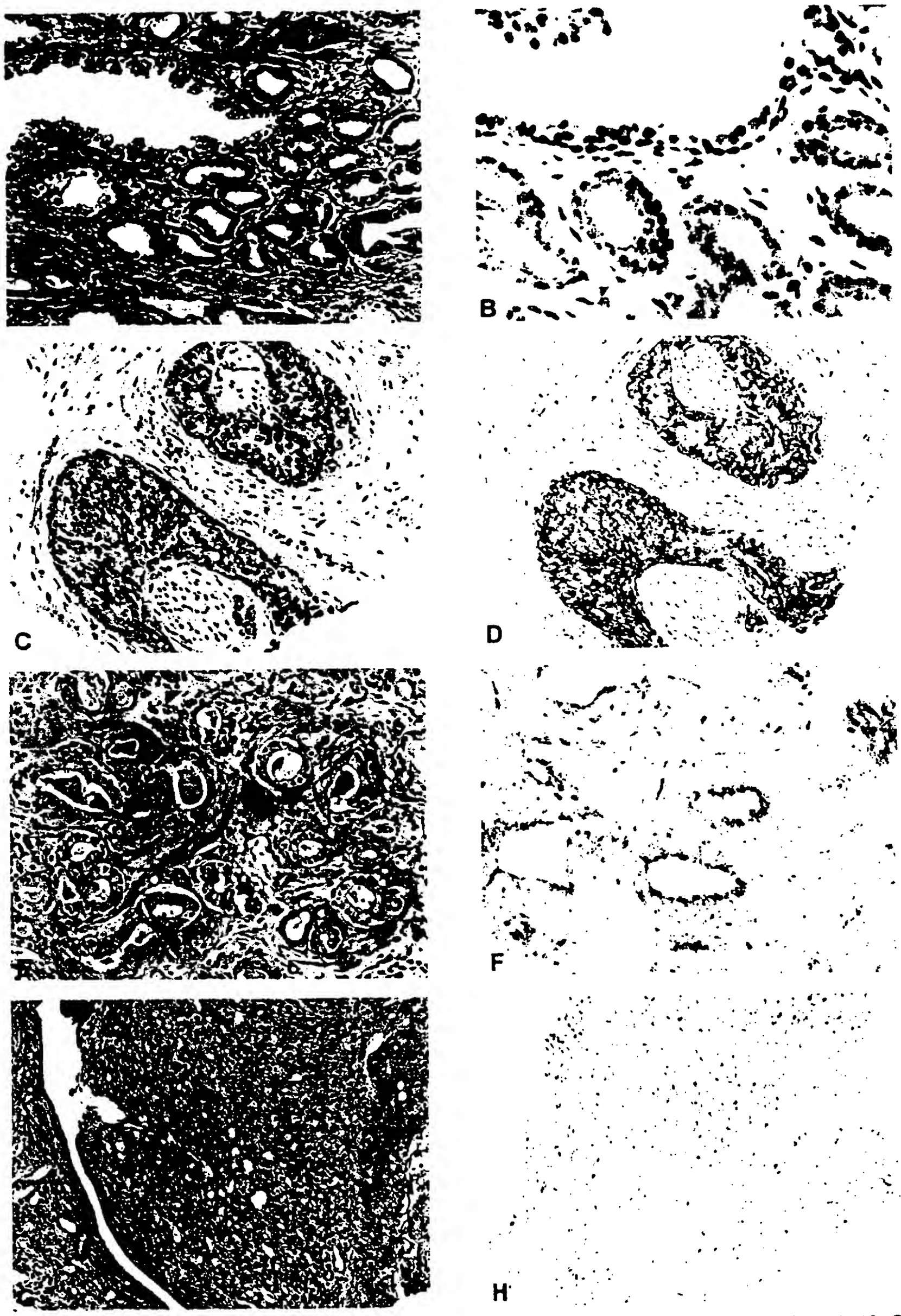

FIG. 1. P504S immunohistochemical staining (**B, D, F, H**) and corresponding hematoxylin and eosin stain (**A, C, E, G**) in cases of conventional prostatic carcinoma. (**A** and **B**) Circumferential luminal staining in a Gleason pattern 3 carcinoma. (**C** and **D**) Diffuse cytoplasmic staining in a Gleason pattern 4 carcinoma with perineural invasion. (**E** and **F**) Circumferential luminal to subluminal staining in a Gleason pattern 3 carcinoma. (**G** and **H**) An example of a Gleason pattern 4 carcinoma with no P504S expression.

TABLE 3. *Correlation of extent of P504S staining in prostate cancer with Gleason score*

Amount of P504S staining	Gleason score			
	6 or less	7	8 or more	p value*
1+, 2+, or 3+	82%	88%	74%	0.201
2+ or 3+	70%	81%	65%	0.156

* χ^2 analysis. p <0.05 considered significant.

of atrophy had focal, faint, and noncircumferential staining (Fig. 5). Seminal vesicles were negative for P504S. However, five of 10 specimens with seminal vesicle epithelium had nonspecific staining that correlated in intensity with the amount of pigment observed in the hematoxylin and eosin-stained slides. Staining in benign epithelium was seen in glands with or without associated inflammation. One specimen diagnosed as "atypical small acinar proliferation suspicious for cancer" had 2+ cytoplasmic staining.

P504S Expression in Prostatic Stroma

In most specimens the fibromuscular stroma of the prostate showed weak diffuse staining. This did not hinder the evaluation and interpretation of staining in prostatic epithelium (Fig. 1D, F).

P504S Expression in Invasive Urothelial Carcinoma

Six of six (100%) specimens (including one TURP specimen) with invasive urothelial carcinoma had at least focal staining. Five of six (83%) specimens had 2+ staining and one specimen had 1+ staining. In general, the staining was fine to granular, cytoplasmic, and moderate to strong in intensity. The TURP specimen with hormonally treated prostate carcinoma and invasive urothelial carcinoma had 2+ and 3+ staining in the prostatic and urothelial carcinomas, respectively (Fig. 6).

DISCUSSION

Prostate cancer continues to be a significant cause of mortality and morbidity in men in the United States.[5] Attempts at the early detection of prostate cancer using serum prostate specific antigen levels and transrectal ultrasound have resulted in increased numbers of patients undergoing 18-gauge needle prostate biopsy. In addition, the number of biopsies on average from each patient has increased.[3,4] Predictably, the advancements in the early detection of prostate cancer in the contemporary era has necessitated making diagnoses with seemingly smaller and smaller amounts of prostate cancer in 18-gauge needle biopsies.

The diagnosis of cancer in 18-gauge needle biopsies is made by use of traditional histologic parameters, including architecture, nuclear features, and the presence or absence of a basal cell layer.[11-15] Immunohistochemistry using monoclonal antibodies against high molecular weight cytokeratins (HMWCK) is used to help confirm small foci of carcinoma, carcinomas with unusual histologic patterns (e.g., pseudohyperplastic, atrophic, ductal), and in prostate biopsies following hormonal therapy or radiation.[15] Although HMWCK immunohistochemistry is an extremely useful adjunctive study, it is important to recognize that the diagnosis of cancer is based on the absence of a detectable HMWCK-positive basal cell layer in prostate carcinoma. However, some morphologically benign glands, foci of atypical adenomatous hyperplasia, and HGPIN can have discontinuous or even absent basal cell layers as detected by HMWCK immunohistochemistry.[2,15] Furthermore, some prostatic carcinomas, especially ductal carcinomas, may have a basal cell layer that is detectable with HMWCK immunohistochemistry.[2,8] Recently, other basal cell-specific antibodies, including cytokeratin 5/6 and p63, have shown similar utility in detecting a basal cell layer in prostatic pathology[1-10] but are not yet widely used currently in clinical practice.

A sensitive and specific "positive" immunohistochemistry marker for prostate cancer would be a valuable diagnostic tool for the modern surgical pathology laboratory. The recent study of P504S by Jiang et al. suggests that P504S may be such a marker.[6] Jiang et al. demonstrated that P504S was expressed at least focally in all cases of prostate carcinoma examined, including those previously treated with radiation, with 92% of cases showing diffuse expression.[6] Interestingly, HGPIN and atypical adenomatous hyperplasia were also found to strongly express P504S, in keeping with the putative precursor potential of these lesions.[6,14] Jiang et al. also found focal and weak staining of benign glands in 12% of cases, although small benign glandular proliferations such as atrophy were consistently negative.[6]

In the current study, 82% of specimens of prostatic carcinoma were P504S positive regardless of Gleason score, and 72.6% had at least 2+ staining. P504S immunostaining was observed in all variants and unusual morphologic patterns of prostatic carcinoma, including ductal (100%) and mucinous (67%) variants and pseudohyperplastic (83%) and atrophic (100%) patterns. Staining amount and pattern in variants and morphologic variations of prostatic carcinoma were similar to that in conventional prostatic carcinoma. Staining was also observed in posttreatment prostatic carcinoma, including both posthormonal (100%) and postradiation (80%) therapy specimens. However, we also observed at least focal staining in 21% of benign prostatic epithelium. Specific benign small gland proliferations, including at-

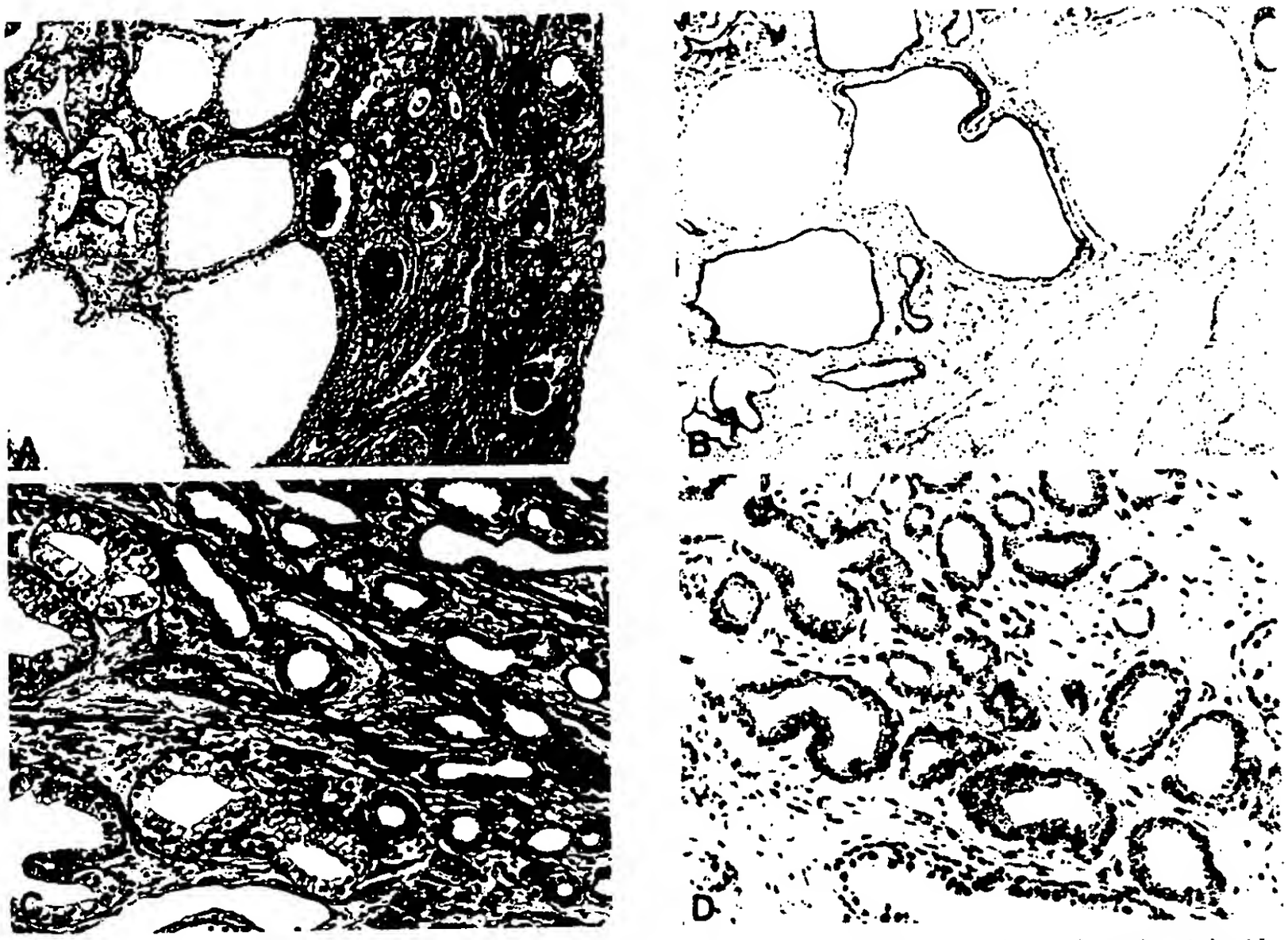

FIG. 2. P504S immunohistochemical staining (**B** and **D**) and corresponding hematoxylin and eosin stain (**A** and **C**) in cases with unusual morphologic patterns of conventional prostatic carcinoma. (**A** and **B**) Circumferential luminal to subluminal staining in a pseudohyperplastic pattern. (**C** and **D**) Circumferential luminal staining in an atrophic pattern.

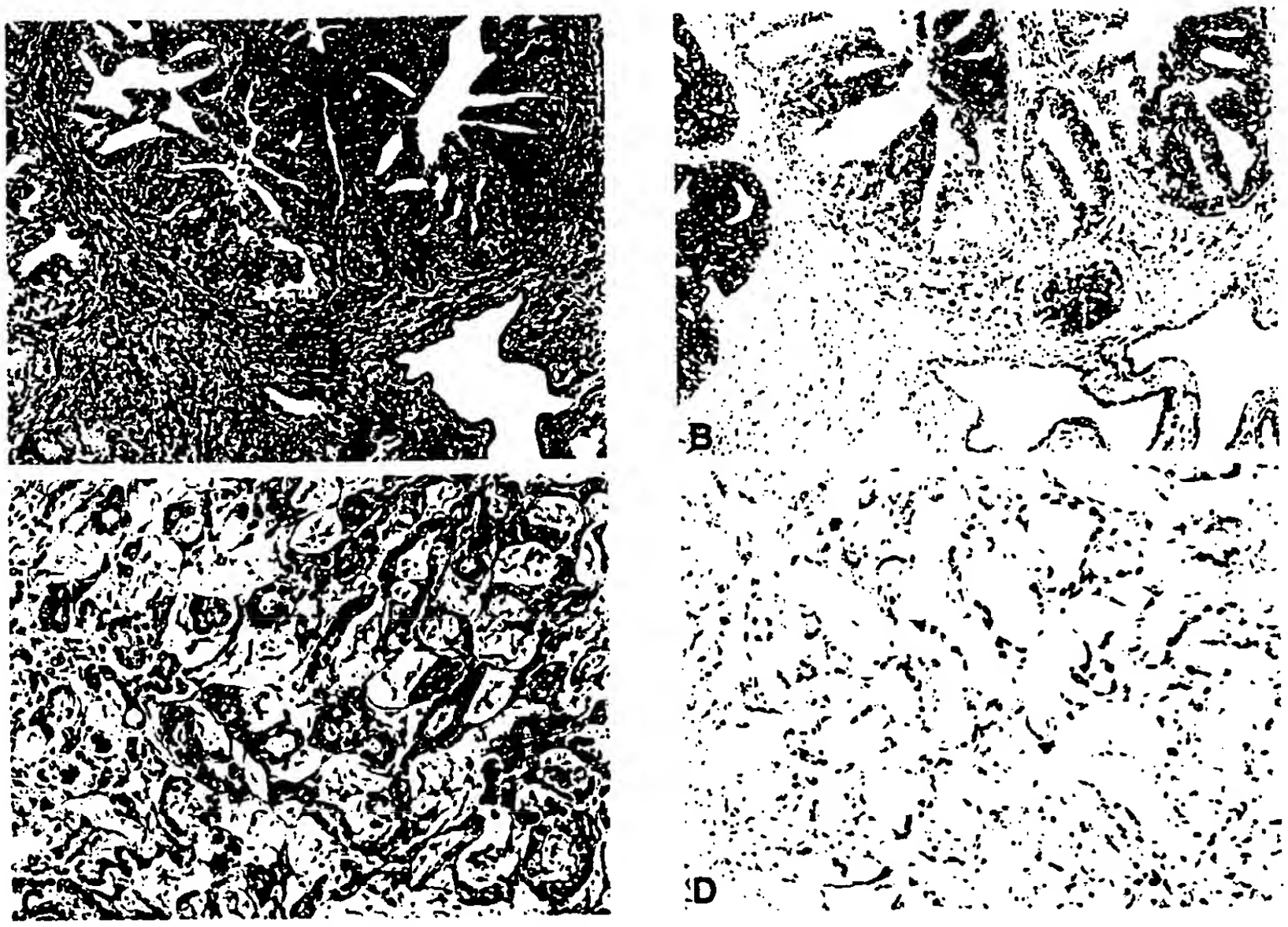

FIG. 3. P504S immunohistochemical staining (**B** and **D**) and corresponding hematoxylin and eosin stain (**A** and **C**) in variants of prostatic carcinoma. (**A** and **B**) Diffuse cytoplasmic staining in a ductal adenocarcinoma (**C** and **D**) Heterogeneous cytoplasmic staining in a mucinous carcinoma.

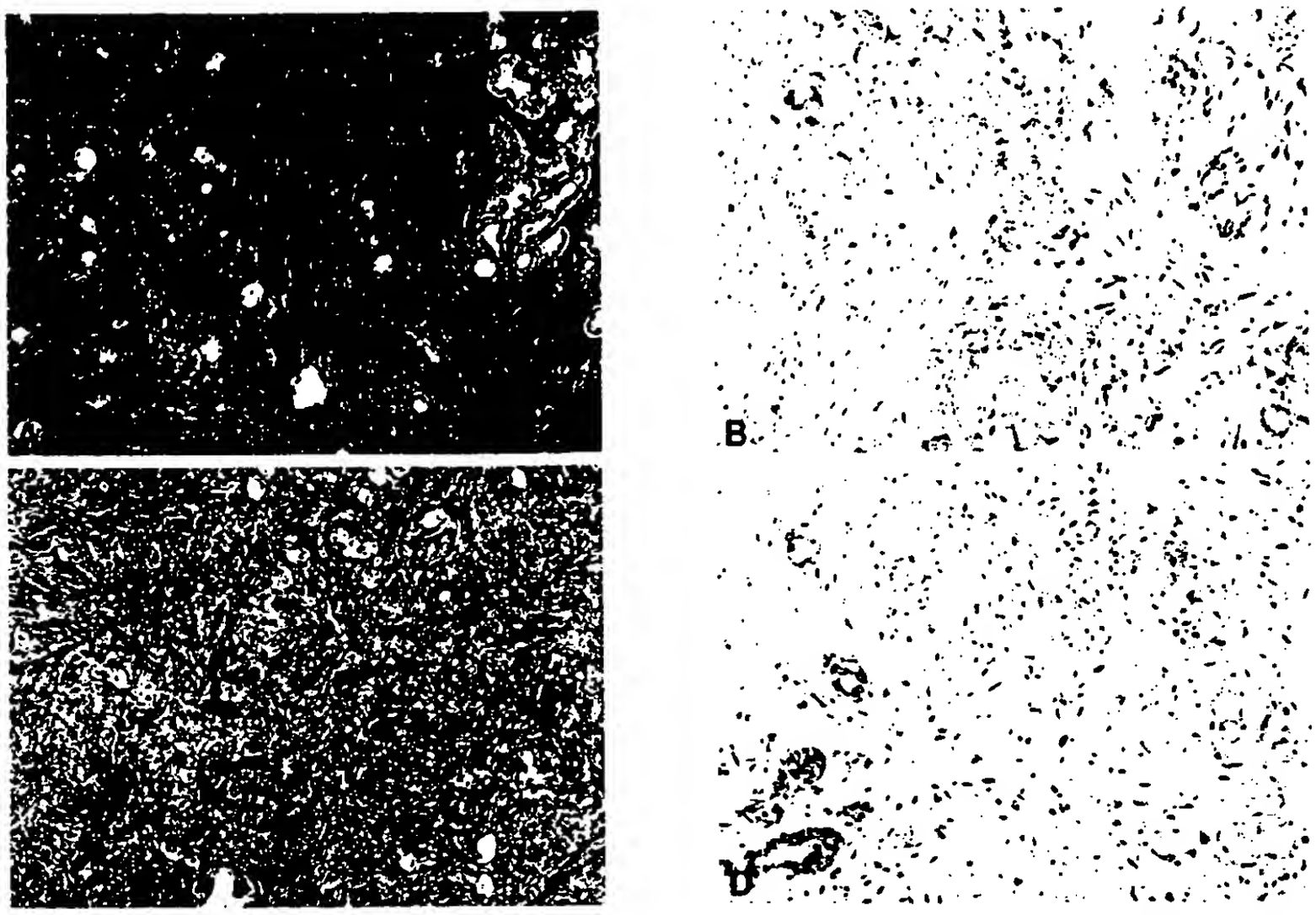

FIG. 4. P504S immunohistochemical staining (**B** and **D**) and corresponding hematoxylin and eosin stain (**A** and **C**) in posttherapy carcinomas. (**A** and **B**) Posthormonal therapy. (**C** and **D**) Postradiation therapy.

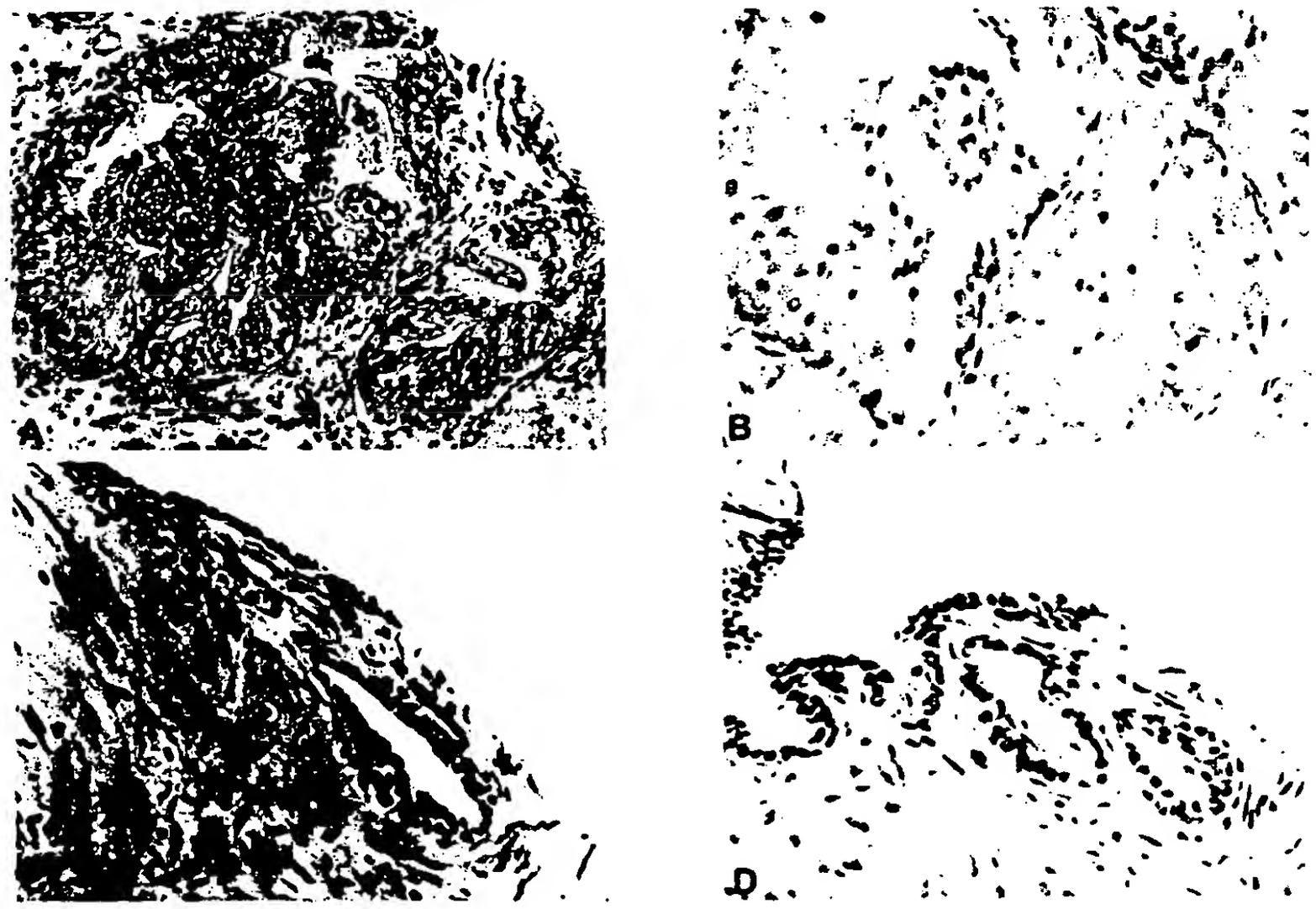

FIG. 5. P504S immunohistochemical staining (**B** and **D**) and corresponding hematoxylin and eosin stain (**A** and **C**) in cases demonstrating P504S expression in benign prostatic tissues. (**A** and **B**) Focal, noncircumferential staining in benign prostatic glands with no specific morphologic pattern. (**C** and **D**) Focal, noncircumferential staining in atrophic glands. Note the weak staining in prostatic stroma.

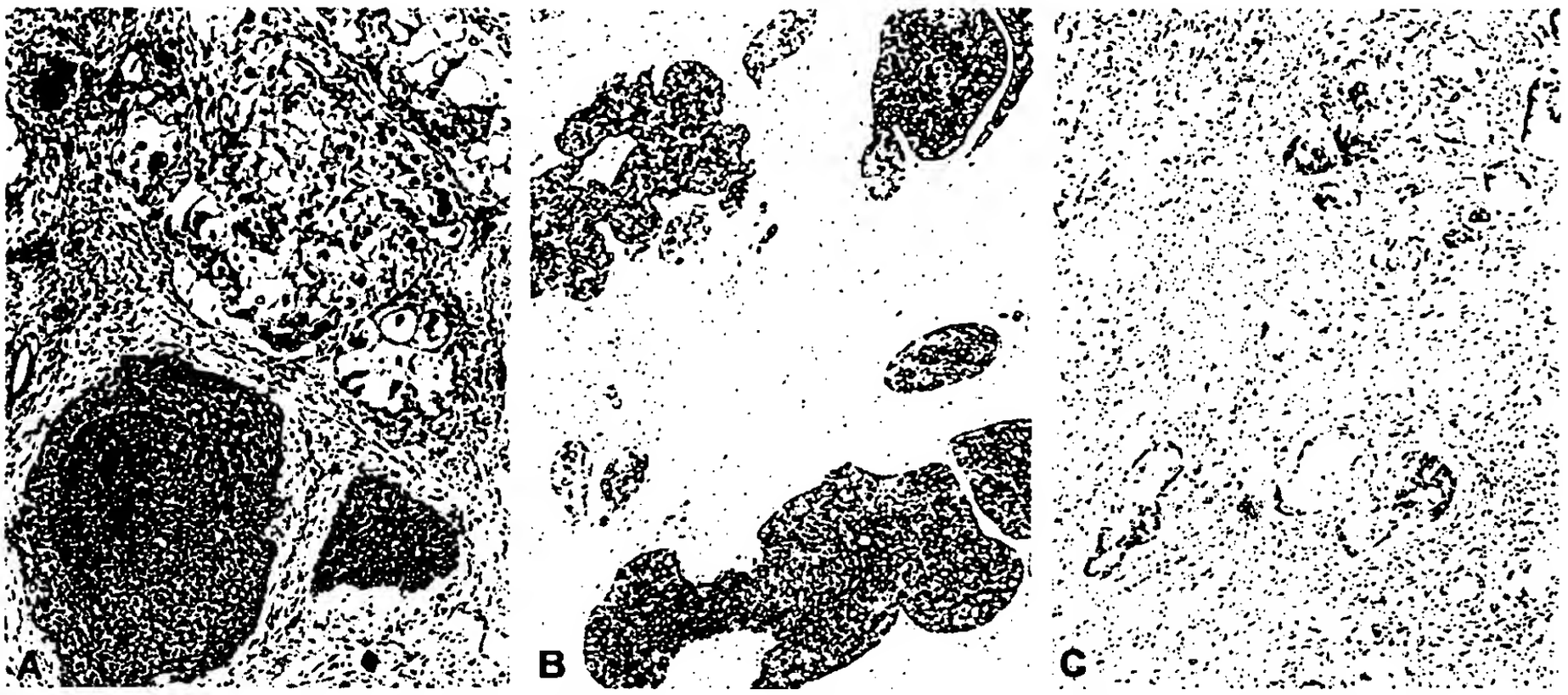

FIG. 6. P504S immunohistochemical staining (**B** and **C**) and corresponding hematoxylin and eosin stain (**A**) in a TURP specimen with concomitant urothelial carcinoma (**A**, lower left, and **B**) and hormonally treated prostatic carcinoma (**A**, upper right, and **C**).

rophy, postatrophic hyperplasia, transitional metaplasia, and basal cell hyperplasia, were negative except for rare foci of atrophy with minimal staining. It must be noted, however, that sclerosing adenosis and atypical adenomatous hyperplasia were not represented in our series of cases, nor are there data regarding the staining in the former in the literature.

It is important to recognize that P504S expression is not limited to carcinomas of prostatic origin. In this study we found P504S expression in all studied specimens of invasive urothelial carcinoma. In addition, HGPIN has been shown to be positive for P504S in at least 38.5% of specimens.[13] P504S expression has recently been documented in normal liver and kidney tissue as well as a variety of malignant neoplasms, including hepatocellular, colorectal, renal cell, and gastric carcinomas.[7]

Our study confirms the diagnostic utility of P504S in the diagnosis of prostate cancer from 18-gauge needle biopsies. We think that the finding of intense, circumferential luminal, subluminal, or cytoplasmic P504S expression in a focus morphologically suspicious for carcinoma supports the diagnosis of prostatic cancer, much in the same way that the finding of an absent basal cell layer by HMWCK immunohistochemistry does. There are several possible indications for P504S immunohistochemistry in prostate needle biopsies: adjunctive confirmation of extremely small foci of carcinoma; cases with small acinar proliferations in which the differential diagnosis includes carcinoma, atrophy, and post atrophic hyperplasia, etc.; specimens with carcinoma that have been treated with hormone or radiation therapy; and specimens with unusual morphologic patterns or variants of prostate cancer, including atrophic, pseudohyperplastic, ductal, and mucinous. It is unclear as to what extent P504S will be valuable in cases currently diagnosed as "atypical small acinar proliferation" that fall short of the morphologic threshold for the diagnosis of cancer. Also, our analysis of postradiation and posthormonal therapy-treated cases should be regarded as preliminary, and proof of principle analysis and additional detailed studies in these specific clinical settings assessing the utility of P504S are warranted.

In summary, our study supports and expands upon the earlier findings of Jiang et al.[6] with regard to the use of P504S immunohistochemistry in the diagnosis of prostatic carcinoma. Although we found P504S to be a sensitive marker of prostatic carcinoma, it is critical to remember that it may be negative in a subset of carcinomas and positive in some benign glands. As with any immunohistochemical reagent, P504S must therefore be used only in the context of strict morphologic correlation. Furthermore, we think that it is prudent to always use P504S along with the better established negative marker HMWCK because the contrasting immunostaining patterns for carcinoma (staining and lack of staining with P504S and HMWCK immunohistochemistry, respectively) compliment each other and compound the diagnostic confidence. The concurrent use of these two antibodies is also important, as P504S immunostaining alone will not help distinguish between high-grade prostate intraepithelial neoplasia and a ductal pattern of prostatic adenocarcinoma or between atypical adenomatous hyperplasia and prostatic adenocarcinoma. The combination of the findings of HMWCK and P504S would be important in these differential diagnoses. Because

HMWCK and possibly P504S immunohistochemistry may contribute to the diagnosis of prostate cancer in any given case, processing prostate biopsies so that levels 1, 3, and 5 are used for hematoxylin and eosin staining and levels 2 and 4 are set aside for potential immunohistochemistry or additional hematoxylin and eosin staining may be highly beneficial.

Acknowledgments

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α-Methylacyl-CoA Racemase: Expression Levels of this Novel Cancer Biomarker Depend on Tumor Differentiation

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α-Methylacyl-CoA racemase (AMACR) has previously been shown to be a highly sensitive marker for colorectal and clinically localized prostate cancer (PCa). However, AMACR expression was down-regulated at the transcript and protein level in hormone-refractory metastatic PCa, suggesting a hormone-dependent expression of AMACR. To further explore the hypothesis that AMACR is hormone regulated and plays a role in PCa progression AMACR protein expression was characterized in a broad range of PCa samples treated with variable amounts and lengths of exogenous anti-androgens. Analysis included standard slides and high-density tissue microarrays. AMACR protein expression was significantly increased in localized hormone-naive PCa as compared to benign ($P < 0.001$). Mean AMACR expression was lower in tissue samples from patients who had received neoadjuvant hormone treatment but still higher compared to hormone-refractory metastases. The hormone-sensitive tumor cell line, LNCaP, demonstrated stronger AMACR expression by Western blot analysis than the poorly differentiated cell lines DU-145 and PC-3. AMACR protein expression in cells after exposure to anti-androgen treatment was unchanged, whereas prostate-specific antigen, known to be androgen-regulated, demonstrated decreased protein expression. Surprisingly, this data suggests that AMACR expression is not regulated by androgens. Examination of colorectal cancer, which is not hormone regulated, demonstrated high levels of AMACR expression in well to moderately differentiated tumors and weak expression in anaplastic colorectal cancers. Taken together, these data suggest that AMACR expression is not hormone-dependent but may in fact be a marker of tumor differentiation. *(Am J Pathol 2002, 161:841–848)*

Prostate cancer (PCa) is the most common non-skin cancer diagnosed in men in the United States.[1] One explanation for the rapid increase in the incidence of PCa diagnosis has been the advent of prostate-specific antigen (PSA) screening. PSA screening has led to earlier detection of PCa.[2] However, the impact of PSA screening on cancer-specific mortality is still unknown pending the results of prospective randomized screening studies.[3–5] A major limitation of the serum PSA test is lack of PCa sensitivity and specificity especially in the intermediate range of PSA detection (4 to 10 ng/mL). Our group has concentrated on developing and validating novel PCa biomarkers using a combined expression and tissue microarray (TMA) approach.[6] This approach by our group and others has led to the identification of hepsin, a serine protease, up-regulated in PCa.[7–10] Furthermore, our group was able to use high-density TMAs to determine associations of hepsin protein and another protein, pim-1 kinase, with clinical outcome.[6]

Using a similar approach, α-methylacyl-CoA racemase (AMACR), an enzyme that plays an important role in bile acid biosynthesis and β-oxidation of branched-chain fatty acids,[11–13] was also recently identified. AMACR was determined to be up-regulated in PCa after examination of several independent gene expression data sets, including our own.[14,15] These findings were subsequently confirmed by different groups on the protein level, even when using different types of antibodies for immunoblot analysis and high-density TMAs.[16–18] Interestingly, hormone-refractory metastatic PCa demonstrated lower AMACR expression than hormone-naive PCa. This observation suggested that AMACR protein expression is regulated by androgens. It is extremely important to identify PCa biomarkers, which portend an aggressive clinical course, given that hormone-refractory tumors are virtually all e-

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that. However, currently no clinical marker is available to identify a subgroup of localized tumors that may eventually develop into lethal PCa. To examine the intriguing possibility that the PCa biomarker AMACR might play a role in hormone dysregulation of localized PCa, we undertook the current study.

Materials and Methods

Sample Collection, cDNA Array, and TMA Construction and Evaluation

Clinical samples were taken from the Radical Prostatectomy Series and from the Rapid Autopsy Program at the University of Michigan.[16] Both are part of the University of Michigan Prostate Cancer Specialized Program of Research Excellence (SPORE). Primary PCa of metastatic cases as well as lymph node metastases were contributed in collaboration from the University of Ulm, Ulm, Germany. Detailed clinical expression analyses as well as TMA data were acquired and are maintained on a secure relational database[17] according to the Institutional Review Board protocol of both institutions.

Tissue procurement for expression analysis on RNA level was described in detail elsewhere.[6] For the development of TMA, samples were embedded in paraffin. The study pathologist (MAR) reviewed slides of all cases and circled areas of interest. These slides were used as a template for construction of the six TMAs used in this study. All TMAs were assembled using the manual tissue arrayer (Beecher Instruments, Silver Spring, MD). At least three tissue cores were sampled from each donor block. Histological diagnosis of the tissue cores was verified by standard hematoxylin and eosin (H&E) staining of the initial TMA slide. Standard biotin-avidin complex immunohistochemistry was performed using a polyclonal anti-AMACR antibody (kind gift of Ronald J. A. Wanders, University of Amsterdam, Amsterdam, The Netherlands). Digital images were acquired using the BLISS Imaging System (Bacus Laboratory, Lombard, IL). Staining intensity was scored as negative (score = 1), weak (score = 2), moderate (score = 3), and strong (score = 4). For exploration of the treatment effect by the means of hormonal withdrawal before radical prostatectomy, standard slides were used for regular H&E staining and consecutive sections for detection of AMACR expression. To test AMACR expression in poorly differentiated colon cancers, cases were used from a cohort of well-described colon tumors.[14] In addition to well-differentiated colon cancers, a recently described subset of poorly differentiated colon carcinomas with a distinctive histopathological appearance, termed "large-cell minimally differentiated carcinomas," was used. As previously described, these poorly differentiated colon carcinomas had a high frequency of the microsatellite instability phenotype.[18]

Cell Culture and Immunoblot Analysis

Prostate cell lines (RWPE-1, LNCaP, PC-3, and DU-145) were obtained from the American Type Culture Collection (Rockville, MD). Cells were maintained in RPMI 1640 with 8% decomplemented fetal bovine serum, 0.1% glutamine, and 0.1% penicillin and streptomycin (BioWhittaker, Walkersville, MD). Cells were grown to 75% confluence and then treated for 24 and 48 hours with the antiandrogen bicalutamide (Casodex, Zeneca Pharmaceutical, Plankstadt, Germany) at a final concentration of 20 μmol/L or with methyltrienolone (synthetic androgen) (R1881; NEN Life Science Products, Boston, MA) at a final concentration of 1 nmol/L. Cells were harvested and lysed in Nonidet P-40 lysis buffer containing 50 mmol/L Tris-HCl, pH 7.4, 1% Nonidet P-40 (Sigma, St. Louis, MO) and complete proteinase inhibitor cocktail (Roche, Indianapolis, IN). Fifteen μg of protein extracts were mixed with sodium dodecyl sulfate sample buffer and electrophoresed onto a 10% sodium dodecyl sulfate-polyacrylamide gel under reducing conditions. After transferring, the membranes (Amersham Pharmacia Biotech, Piscataway, NJ) were incubated for 1 hour in blocking buffer (Tris-buffered saline with 0.1% Tween and 5% nonfat dry milk). The AMACR antibody (kind gift of Dr. Wanders) was applied at 1:10,000 diluted blocking buffer overnight at 4°C. After three washes with TBS-T buffer, the membrane was incubated with horseradish peroxidase-linked donkey anti-rabbit IgG antibody (Amersham Pharmacia Biotech) at 1:5000 for 1 hour at room temperature. The signals were visualized with the ECL detection system (Amersham Pharmacia Biotech, Piscataway, NJ). For β-tubulin blots, membranes were stripped with Western Re-Probe buffer (Geno-tech, St. Louis, MO) and blocked in Tris-buffered saline with 0.1% Tween with 5% nonfat dry milk, and incubated with rabbit anti-β-tubulin antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) at 1:500 for 2 hours. For PSA expression the membranes were reprobed in the described manner with PSA antibody (rabbit polyclonal; DAKO Corporation, Carpinteria, CA) at 1:1000 dilution and further processed.

Statistical Analysis

Primary analysis of the cDNA expression data were done with the Genepix software. Cluster analysis with the program Cluster and generation of figures with TreeView was performed as described earlier.[7] AMACR protein expression was statistically evaluated using the mean score result for each prostate tissue type (ie, benign prostate, naive localized or advanced PCa, hormone-treated and hormone-refractory PCa). To test for significant differences in AMACR protein expression between all tissue types, we performed a one-way analysis of variance test. To determine differences between all pairs a post hoc analysis using the Scheffe method was applied as described earlier.[14] For comparison of naive primaries to their corresponding lymph node metastases with respect to AMACR protein expression, a nonparametric analysis (Mann-Whitney test) was performed. To compare AMACR expression intensity to the scored hormonal effect of the pretreated localized PCa cases the Mantel-Haenszel chi-square test was applied. AMACR expression scores are presented in a graphical format using error bars with 95%

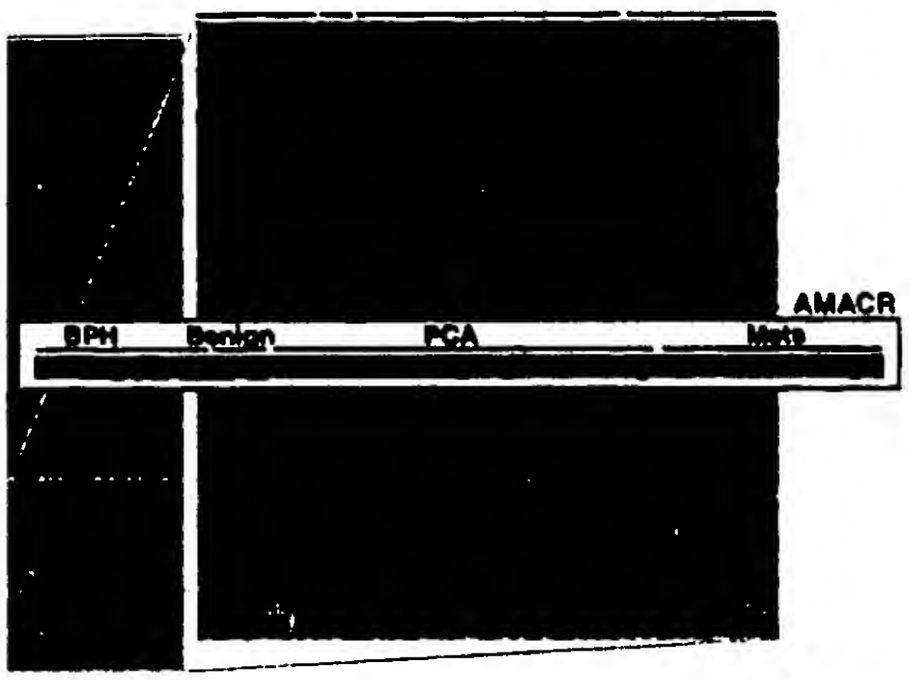

Figure 1. Seventy-six prostate tissue samples including benign prostate, benign prostatic hyperplasia, localized PCa, and metastatic PCa, were analyzed using cDNA expression array analysis. Hierarchical clustering was performed, filtering for only those genes with at least a 1.5-fold expression difference compared to a pool of normal prostate. AMACR was one of the genes that was found to be significantly overexpressed in PCa. Data given are a measure of relative gene expression of AMACR in each sample. Red and green represent up- and down-regulation, respectively. Lighter shading correlates with increased expression.

confidence intervals (CIs). *P* values <0.05 were considered statistically significant.

Results

Hierarchical clustering of 76 prostate tissues including benign prostate, benign prostatic hyperplasia, localized PCa, and metastatic PCa and filtering for only those genes with a 1.5-fold expression difference or greater, clustered the samples into histologically distinct groups as previously described.[6] As demonstrated by a TreeView presentation of this data (Figure 1), AMACR was one of several genes that demonstrated overexpression at the cDNA level of PCa samples with respect to benign pooled prostate tissue. Interestingly, although some of the hormone-refractory cases demonstrated overexpression, as depicted by the red shading, the highest level of overexpression by cDNA analysis was in the clinically localized PCa cases.

To further investigate the role of AMACR protein expression in samples with variable differentiation and exposure to anti-androgen treatment, several TMAs with a wide range of PCa were constructed: a total of 119 benign prostate samples, 365 primary hormone-naive PCa samples, 37 naive PCa lymph node metastases, and 41 hormone-refractory metastatic PCa samples were evaluated. An additional 49 hormone-treated primary PCas (including 22 on standard slides) were examined for histological changes associated with anti-androgen treatment and AMACR protein expression. In our hands the percentage of stained cancer cells per sample was 95% in the cases studied. This was independent from the scored intensity of AMACR staining. The mean AMACR protein expression levels for each tissue category is presented in Figure 2A. Benign prostate, naive primary PCa, hormone-treated primary cancer, and hormone-refractory metastatic tissue had a mean staining intensity of 1.28 [standard error (SE), 0.038; 95% CI, 1.20 to 1.35], 3.11 (SE, 0.046; CI, 3.02 to 3.20), 2.86 (SE, 0.15; CI, 2.56 to 3.15), and 2.52 (SE, 0.15; CI, 2.22 to 2.28), respectively. One-way analysis of variance analysis revealed a *P* value of <0.0001. To specifically examine the difference between different tissue types, a post hoc pair-wise comparison was performed. Clinically localized PCa demonstrated a significantly stronger AMACR protein expression as compared to benign prostate tissue (post hoc analysis using Scheffé method; mean difference = 1.83; $P < 0.0001$; CI, 1.53 to 2.13). As expected from previous work, a significant decrease in AMACR protein expression was observed in the metastatic hormone-refractory PCa samples with respect to clinically localized PCa (0.59, $P = 0.002$; CI, 0.15 to 1.03). Hormone-treated primaries had a mean AMACR expression of 2.86, which was between the expression levels of naive primaries (3.11) and hormone-refractory cases (2.52) (post hoc analysis using Scheffé method; $P = 0.51$; CI, −0.66 to 0.16; and $P = 0.56$; CI, −0.23 to 0.91). Interestingly there was no significant difference in AMACR expression in the 37 naive primary prostate samples and lymph node metastases derived from the same patient (Mann-Whitney test, $P = 0.8$). In other words, matched primaries and lymph node metastases showed a similar AMACR expression pattern (Figure 2B). Examples of *in situ* AMACR protein expression in a naive primary PCa and a naive lymph node metastasis are presented in Figure 2.

Because there seems to be a wider variability in AMACR protein expression in the hormone-treated PCa samples as compared to naive cases (Figure 2), we further investigated this observation to correlate the hormone treatment effect with AMACR protein expression. We examined a subset of 22 PCa cases in which the patients received variable amounts and types of anti-androgen treatment before surgery. These cases were evaluated blindly with respect to treatment protocol for histological evidence of hormone treatment (H&E slide) and AMACR protein expression. The hormonal effect visible on the H&E slides was classified from 1 to 4 with 1 representing "no effect" and 4 showing a "very strong effect." Thirteen cases demonstrated either no or moderate hormonal effect and nine cases had a very strong hormonal effect. Statistical analysis revealed a significant difference between these two groups with respect to AMACR expression intensity (Figure 3, Mantel-Haenszel chi-square, $P = 0.009$). Figure 3 presents an example of a PCa case treated before surgery with anti-androgens, which has a strong hormonal effect appreciated on H&E and decreased AMACR protein expression (Figure 3A). In these cases, no association was detected between the type of hormone treatment (ie, monotherapy or complete hormonal withdrawal for hormone deprivation) or duration of treatment and AMACR expression.

For further exploration of the hormonal effect on AMACR expression, primary cell culture experiments and Western blot analysis were performed. As demonstrated in Figure 3B, LNCaP cells, derived from a metastatic lesion but considered hormone responsive, showed a higher baseline AMACR expression as compared to PC-3

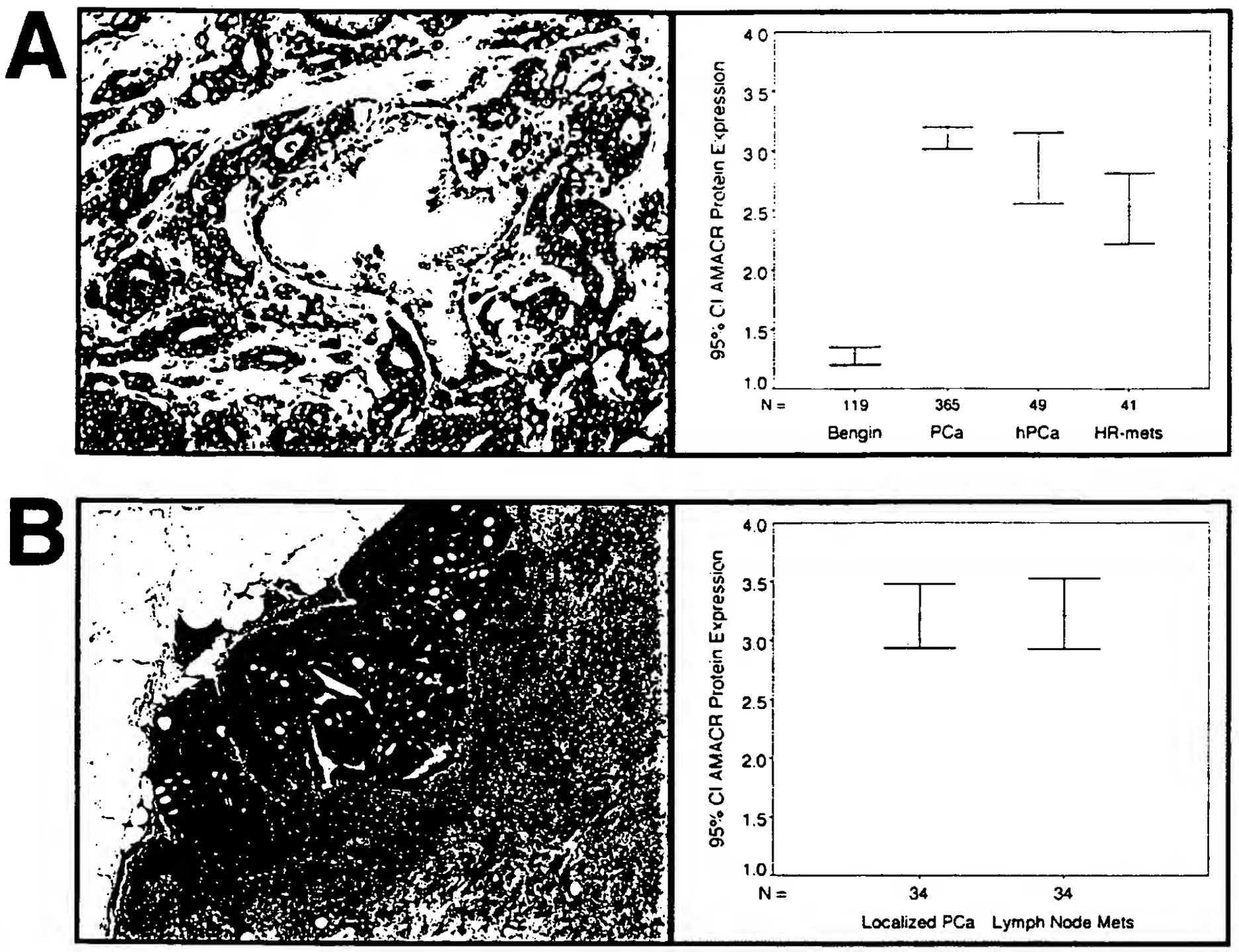

Figure 2. A: AMACR protein expression in localized hormone-naive PCa. The specificity of AMACR is demonstrated by the near complete absence of AMACR expression in benign prostate glands (**center**). Error bars (**right**) representing the 95% CI of the mean expression level of AMACR for the four tissue types: benign prostate (benign), localized hormone-naive PCa (PCa), hormone-treated localized PCa (hPCa), and hormone-refractory distant PCa metastases (HR mets). **B:** Strong AMACR expression in a naive lymph node metastasis. Error bars (**right**) representing the 95% CI of the mean expression of the primary naive PCa and corresponding lymph node metastases. Original magnifications, ×400.

and DU-145 cells, which are both hormone-independent cell lines derived from metastatic lesions. A benign cell line, RWPE-1,[19] showed near absent AMACR expression, which is consistent with our *in situ* protein expression data. To simulate an anti-androgen treatment, we used the hormone-responsive cell line, LNCaP, and treated the cells with bicalutamide in a final concentration of 20 μmol/L for a time period of 24 and 48 hours. Interestingly, AMACR expression in cell lysates of LNCaP cells did not change at either time point when exposed to anti-androgen therapy. Under the same conditions, PSA, a gene known to be regulated by the androgen receptor, showed decreased protein expression. In addition, when LNCaP cells were exposed to a synthetic androgen R1881, no increase in AMACR expression was observed (Figure 3B). Therefore, these cell culture experiments provide evidence that AMACR expression may not be directly regulated by the androgen pathway.

However, another explanation for these observations was that AMACR overexpression occurred in PCa, but as these tumors became poorly differentiated, as in the hormone-refractory PCa, AMACR expression was down-regulated either directly or indirectly because of the process of dedifferentiation. To elucidate this potential correlation we examined colon cancer samples for AMACR expression. As we have recently identified,[20] AMACR protein expression is also observed in some other tumor types, with the highest overall expression in colorectal cancers. Colorectal cancers are not known to be regulated by androgens and therefore represent a good control to test this hypothesis. Twenty well-differentiated and nine anaplastic colon cancer samples were chosen. As previously described, the poorly differentiated tumors have distinct molecular alterations distinguishing them from the common well to moderately differentiated colorectal tumors.[18] Figure 4 demonstrates strong AMACR protein expression in a moderately differentiated colon cancer. This tumor still forms well-defined glandular structures. The surrounding benign colonic tissue does not express AMACR. The anaplastic colon cancers demonstrated weak AMACR protein expression (Figure 4). Moderate to strong AMACR expression was seen in 18 of 20 differentiated cases but in only 2 of 9 anaplastic colonic cancers (chi-square, $P < 0.001$).

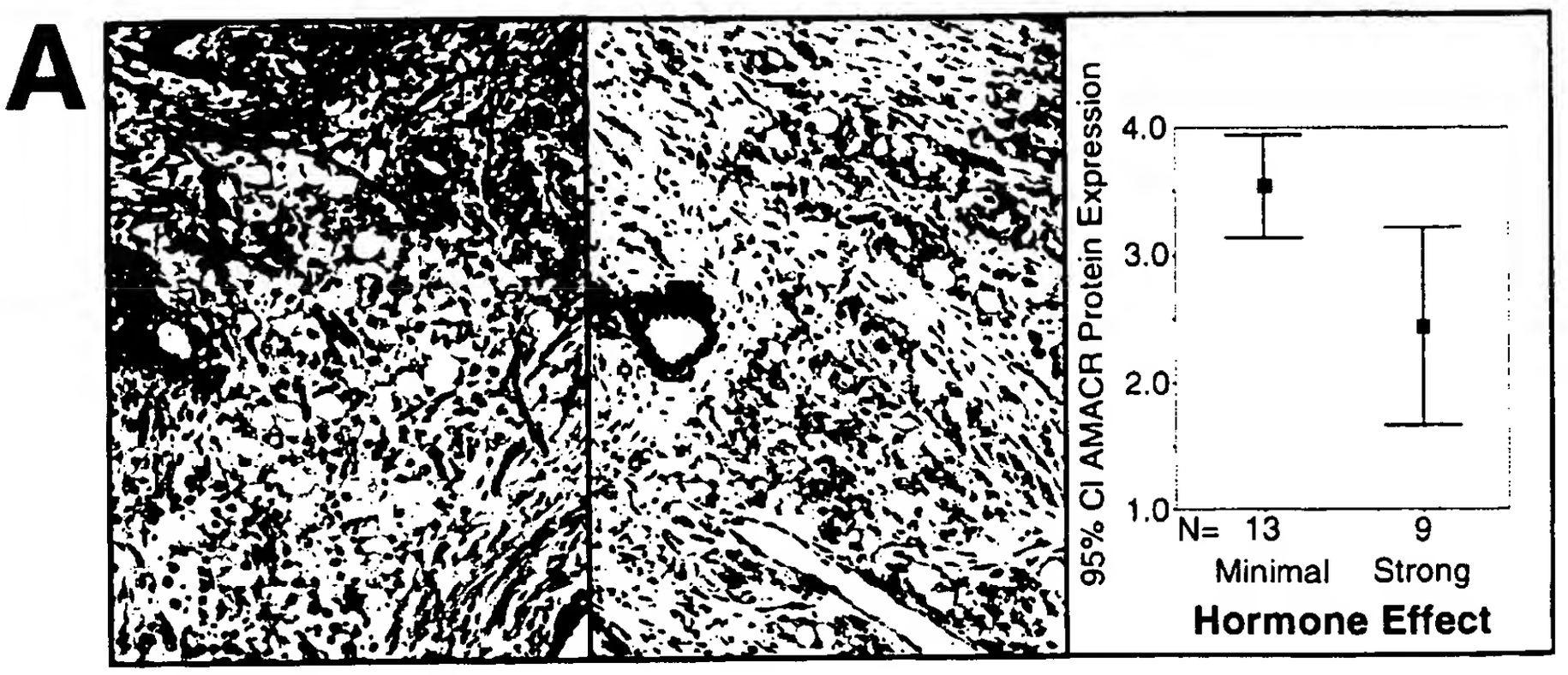

Figure 3. A: PCa demonstrating strong hormonal effect (**right**) because of anti-androgen treatment (H&E). Weak AMACR expression (**middle**) seen in the corresponding immunostained section. Comparison of the mean AMACR expression between PCa demonstrating minimal and strong hormonal effects by H&E examination (**right**, 95% CI). **B: Left**: Western blot analysis representing the baseline AMACR expression in different prostate cell lines. Internal loading control with β-tubulin. **Right**: Western blot analysis of LNCaP cells for AMACR and PSA expression after treatment with an androgen or an anti-androgen for 24 hours and 48 hours. Results of the nontreated control samples (C) and the hormone-treated (T) cells are shown. Original magnification, ×200 (**A**).

Seven of nine of the anaplastic colon cancers had weak to moderate expression. For comparative purposes examples of metastatic hormone-refractory PCa from the TMA experiments are shown in Figure 4. Both of these cases demonstrate weak AMACR protein expression.

Discussion

High-throughput technologies such as cDNA microarrays and TMA have opened a gateway for research. The combination of a tool for discovery with one for verification allows surveying target genes in a very efficient manner. By exploring gene expression data it could be shown that AMACR is consistently overexpressed in PCa.[14] These findings are supported by AMACR expression data described recently in at least two more independent studies using cDNA microarrays.[8,10] Even combined analysis across the three studies revealed statistically significant differential expression of AMACR between benign prostate and PCa.[14] This study focuses on the AMACR expression in different groups of PCa including the aspect of neoadjuvant hormonal withdrawal in localized disease. The most interesting finding in this context was that AMACR expression is decreased in hormone-refractory metastatic tissue samples compared to localized PCa. From the clinical point of view, this observation is very important because localized PCa can be controlled well by different means such as surgery or radiotherapy or even watchful waiting.[8,1,22] On the other hand, there is no consensus as how to treat metastatic disease successfully to positively impact life expectancy.[23] Therefore, identifying PCa biomarkers that are up-regulated in localized PCa but decrease again in far advanced hormone-refractory PCa could help to understand the mechanism involved with the ultimate goal of identifying new treatments.

A surprising finding is that AMACR expression seemed to be hormone-independent in the setting of our cell culture experiments. PSA, a gene known to be regulated by androgens, demonstrated hormone-related alterations in expression under the same conditions. These findings may provide evidence that AMACR is not regulated by the androgen pathway. Still, because we see a de-

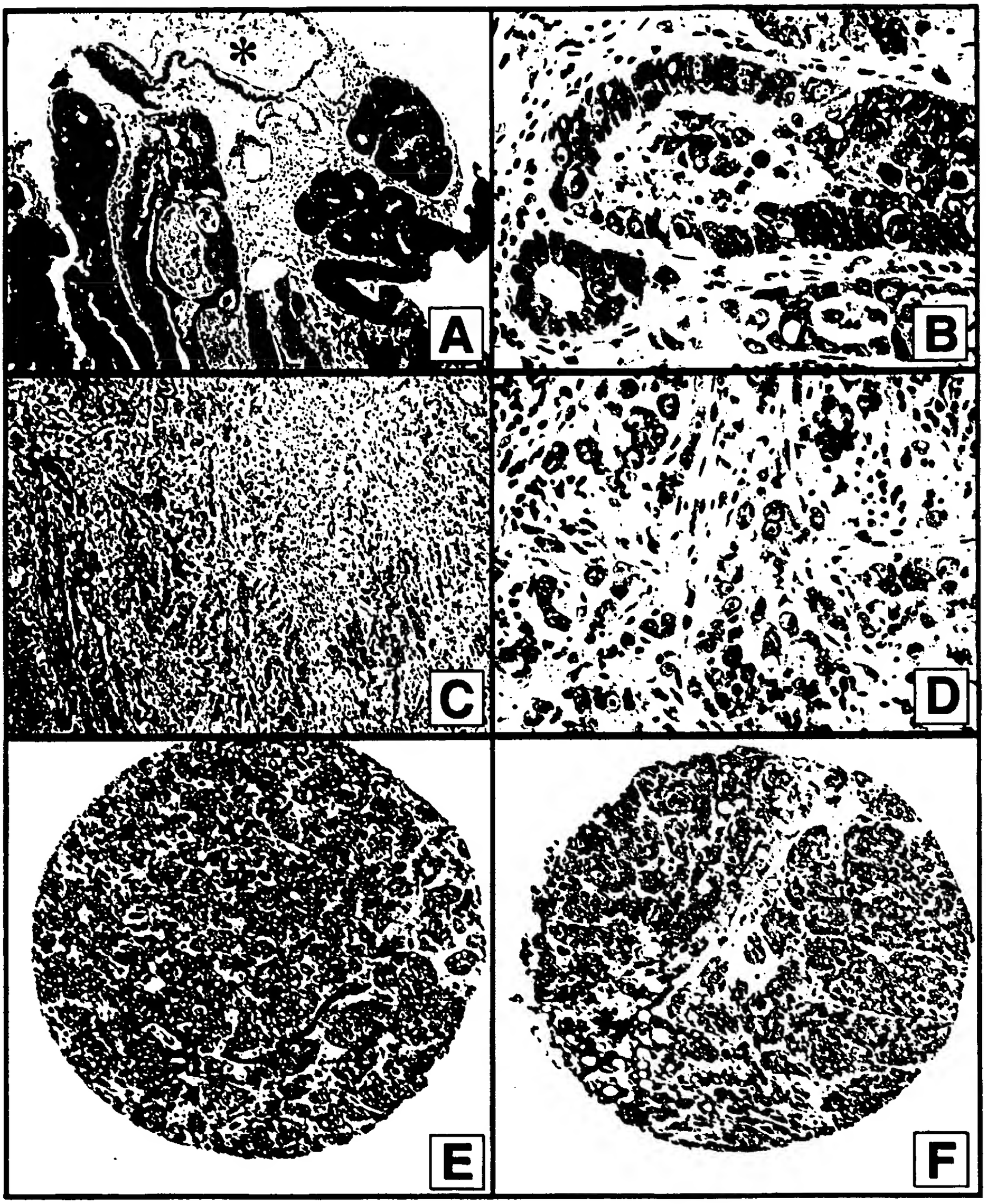

Figure 4. Top: AMACR expression in well-differentiated colon cancer. Cancer specificity demonstrated by the lack of staining in benign colonic glands (*). **Middle**: Weak AMACR expression in an anaplastic colon cancer case. **Bottom**: Weak AMACR expression in representative hormone-refractory PCa metastases. Original magnifications: ×400 (**A**, **C**); ×1000 (**B**); ×100 (**D**); ×200 (**E** and **F**).

creased AMACR expression in hormone-refractory tissue, it might be postulated that AMACR could play a role as a biomarker for hormone resistance. Considering the fact that hormone treatment in the mean of hormonal withdrawal did not affect AMACR expression in the cell culture, may lead to the conclusion that some other-mechanism than the androgen pathway is responsible for AMACR down-regulation in the integrity of cancer tissue.

These findings are considered to be very important and require further investigation.

An alternative hypothesis would suggest that AMACR is overexpressed in the development of cancer, perhaps playing an important role in providing energy for the neoplastic cells. However as the tumors become dedifferentiated they no longer require these sources of energy. Poorly differentiated tumors may take over other pathways to accomplish this same activity of branched fatty acid oxidation. Recent work failed to identify an association with the proliferative rate of the tumor cells and AMACR expression[14] or with the Gleason score and AMACR expression.[15,16] Examination of other tumors demonstrated that colon cancer has the highest AMACR expression (unpublished observations). As colorectal cancers are not known to be hormonally regulated, the fact that dedifferentiation and decreased AMACR expression went hand in hand, further supports the hypothesis that dedifferentiation leads to decreased AMACR expression in the hormone-refractory metastatic PCa. Hormone treatment is also a front line therapy in metastatic PCa but is known to loose efficacy, selecting out hormone-insensitive clones. Anticipating the selection of potentially more dedifferentiated cells because of hormone treatment, an observed strong treatment effect may be consistent with decreased AMACR expression because of the selection of more dedifferentiated cells.

The AMACR gene product is an enzyme that plays an important role in bile acid biosynthesis and β-oxidation of branched-chain fatty acids.[17,18] The link of AMACR expression and neoplasia, however, has only recently been made. AMACR overexpression appears to occur in tumors that have been linked to high-fat diet such as PCa and colorectal cancer.[19] The relationship between fatty acid consumption and cancer is a controversial subject in the development of PCa and colorectal cancer.[20,21] An essential role for AMACR in the oxidation of bile acid intermediates has been demonstrated. AMACR encodes an enzyme that catalyzes the racemization of α-methyl-branched carboxylic acid coenzyme A thioesters and is localized in peroxisomes and mitochondria.[17] As AMACR is involved in the metabolism of lipids it may be speculated that this could due to alterations in the overall balance of lipids. These changes might be associated with DNA damage, malignant transformation and other parameters of cell disturbance. It is still not clear if AMACR is directly involved in cancer progression via a degradative pathway of branched-chain fatty acids or if it represents an epiphenomenon because it is overexpressed in some human tumors. These questions need to be further elucidated.

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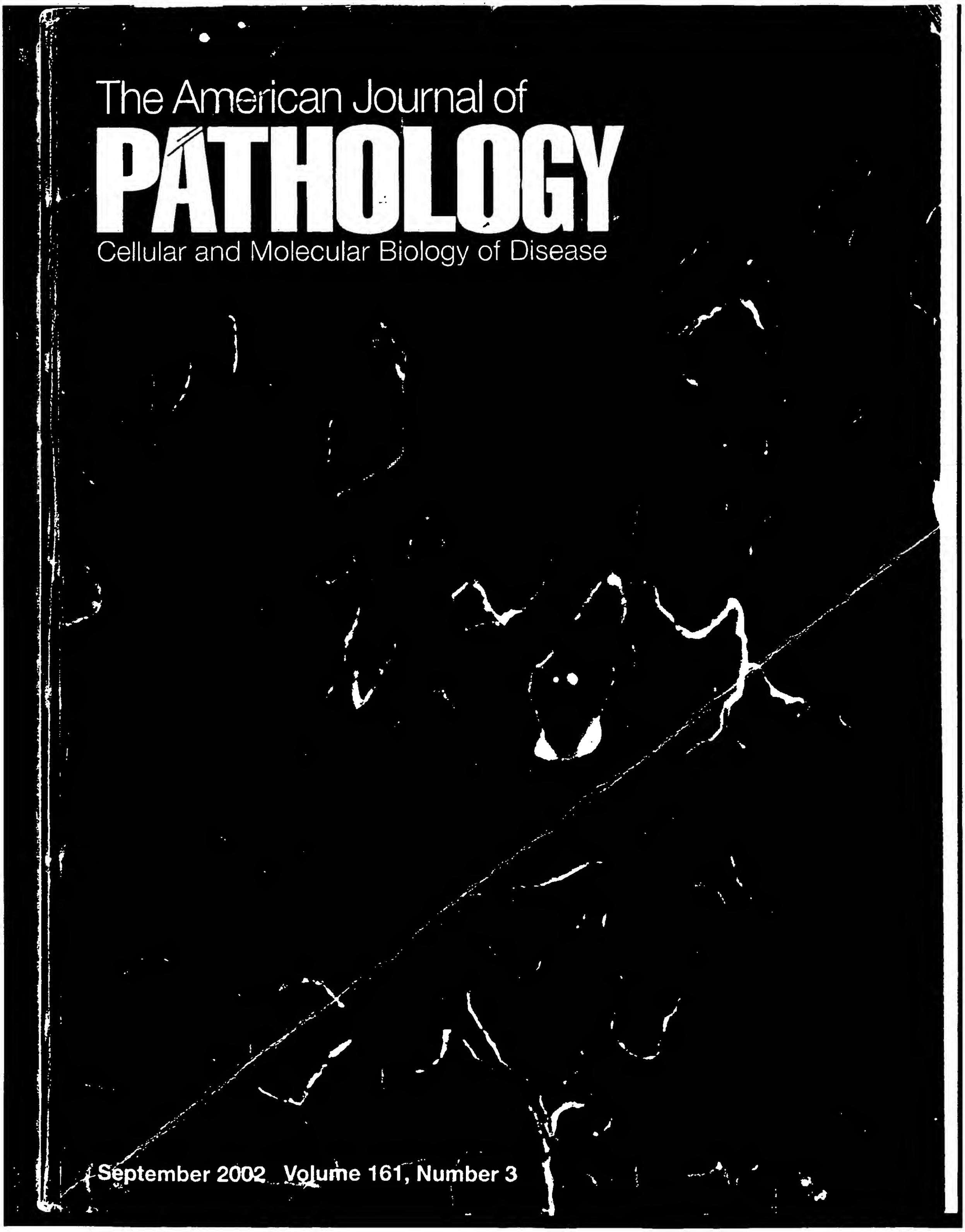

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Exhibit C

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Advances in Brief

α-Methylacyl-CoA Racemase: A New Molecular Marker for Prostate Cancer[1]

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Abstract

Identification of genes that are dysregulated in association with prostate carcinogenesis can provide disease markers and clues relevant to disease etiology. Of particular interest as candidate markers of disease are those genes that are frequently overexpressed. In this study, we describe a gene, *α-methylacyl-CoA racemase* (AMACR), whose expression is consistently up-regulated in prostate cancer. Analysis of mRNA levels of AMACR revealed an average up-regulation of ~9 fold in clinical prostate cancer specimens compared with normal. Western blot and immunohistochemical analysis confirms the up-regulation at the protein level and localizes the enzyme predominantly to the peroxisomal compartment of prostate cancer cells. A detailed immunohistochemical analysis of samples from 168 primary prostate cancer cases using both standard slides and tissue microarrays demonstrates that both prostate carcinomas and the presumed precursor lesion (high-grade prostatic intraepithelial neoplasia) consistently scored significantly higher than matched normal prostate epithelium; 88% of the carcinomas had a staining score higher than the highest score observed for any sample of normal prostate epithelium. Both untreated metastases (n = 32 patients) and hormone refractory prostate cancers (n = 14 patients) were generally strongly positive for AMACR. To extend the utility of this marker for prostate cancer diagnosis, we combined staining for cytoplasmic AMACR with staining for the nuclear protein, p63, a basal cell marker in the prostate that is absent in prostate cancer. In a simple assay that can be useful for the diagnosis of prostate cancer on both biopsy and surgical specimens, combined staining for p63 and AMACR resulted in a staining pattern that greatly facilitated the identification of malignant prostate cells. The enzyme encoded by the *AMACR* gene plays a critical role in peroxisomal β oxidation of branched chain fatty acid molecules. These observations could have important epidemiological and preventive implications for prostate cancer, as the main sources of branched chain fatty acids are dairy products and beef, the consumption of which has been associated with an increased risk for prostate cancer in multiple studies. On the basis of its consistency and magnitude of cancer cell-specific expression, we propose AMACR as an important new marker of prostate cancer and that its use in combination with p63 staining will form the basis for an improved staining method for the identification of prostate carcinomas. Furthermore, the absence of AMACR staining in the vast majority of normal tissues coupled with its enzymatic activity makes AMACR the ideal candidate for development of molecular probes for the noninvasive identification of prostate cancer by imaging modalities.

Introduction

Prostate cancer initiation and progression are processes involving multiple molecular alterations (1). Genomic alterations, combined with changes in the tissue microenvironment, lead inevitably to altered levels of expression of many individual genes in tumor cells. Identification of these genes represents a critical step toward a thorough understanding of prostate carcinogenesis and an improved management of prostate cancer patients. Of particular biological and clinical interest are those genes that are consistently overexpressed in the vast majority of prostate cancers. Such genes and their products, besides providing possibly valuable insight into the etiology of prostate cancer, may have important utility as diagnostic markers in this disease. However, few genes of this nature have been reported to date.

High-throughput gene expression profiling using cDNA microarray allows for systematic interrogation of transcriptionally altered genes. By comparing the mRNA expression profile of cancerous lesions with noncancerous lesions, multiple candidates of molecular markers for prostate cancer have emerged (2–6). One such candidate, the gene for AMACR,[3] was identified as being overexpressed in prostate carcinoma cells when compared with benign or normal prostate epithelial cells (2, 3). AMACR is a well-characterized enzyme (7) that plays a key role in peroxisomal β-oxidation of dietary branched-chain fatty acids and C27-bile acid intermediates. It catalyzes the conversion of (R)-α-methyl-branched-chain fatty acyl-CoA esters to their (S)-stereoisomers. Only the (S)-stereoisomers can serve as substrates for branched-chain acyl-CoA oxidase during their subsequent peroxisomal β-oxidation. Two aspects of this pathway may have particular relevance for prostate carcinogenesis: (*a*) the main sources of branched chain fatty acids in humans (milk, beef, and dairy products; Ref. 8) have been implicated as dietary risk factors for prostate cancer (9); and (*b*) peroxisomal β-oxidation generates hydrogen peroxide (10), a potential source of procarcinogenic oxidative damage (11, 12).

An initial report by Xu *et al.* (2), using a limited number of samples, indicated that AMACR was overexpressed potentially in a subset of prostate cancers at both the mRNA and protein level. Our previous study compared gene expression profiles of 16 prostate cancer and 9 samples of BPH using cDNA microarrays containing 6500 human genes (3). AMACR was expressed highly in the majority of prostate cancer samples, averaging ~6-fold higher levels than the BPH samples. In this study, we expanded these previous studies by including cDNA microarray data from an additional 35 prostate tissue samples, comprising matched normal tumor pairs, and by performing an extensive IHC analysis of both primary and metastatic prostate cancer specimens, including normal and cancerous prostate tissue from 159 patients analyzed on prostate TMAs. Furthermore, we examined the diagnostic utility of combining staining for AMACR with staining for p63, a prostate basal cell marker that is absent in the vast majority of prostate cancers (13, 14). The consistent and extensive up-regulation

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[3] The abbreviations used are: AMACR, α-methylacyl-CoA racemase; IHC, immunohistochemical; KRT8, keratin 8; PIN, prostatic intraepithelial neoplasia; TMA, tissue microarray; BPH, benign prostatic hyperplasia; TBP, TATA-binding protein; GSTP1, glutathione *S*-transferase π; HGPIN, high-grade prostatic intraepithelial neoplasia.

of AMACR that we observe at both the mRNA and protein level strongly suggests that AMACR will be an important new marker for prostate cancer.

Materials and Methods

Prostate Tissue Procurement for cDNA Microarray Analysis. Prostate cancer tissue specimens for cDNA microarray analysis were obtained from 23 patients undergoing radical prostatectomy for clinically localized prostate carcinoma at Johns Hopkins Hospital from 1993 to 2000. Specimens were obtained from the operating room immediately after resection. The seminal vesicles were truncated. If palpable tumor was identified, the specimen was inked and harvested as described previously (15). This results in the banking of the largest palpable tumor, as well as areas of apparent normal and BPH when available. The areas containing tumor that were adjacent to the harvested tumor blocks were submitted for formalin fixation and routine processing. Harvested tissues were flash frozen and stored in −80°C. Paired normal cancer samples were prepared as described previously (3) from 12 of the 23 specimens, whereas only cancer samples were obtained from the other 11 specimens giving 35 samples total for analysis. Cancer samples were microscopically estimated to contain ≥60% (range from 60 to 90%) adenocarcinoma cells in cellular composition, and the normal samples were estimated to contain ≥50% (range from 50 to 75%) epithelial cells. Institutional Review Board-approved informed consent was obtained from all patients in this study.

cDNA Microarray Analysis. RNA extraction, labeling, and hybridization were carried out as described previously (3). A single reference sample composed of a pool of RNA from two BPH specimens was used throughout all hybridizations. Measurement values were extracted for normalized ratios of signal intensities of sample *versus* reference, which represent the relative mRNA abundance for each gene in each sample when compared with the common reference (3).

Real-time Reverse Transcription-PCR. Quantitative PCR was performed on iCycler (Bio-Rad, Richmond, CA) with gene-specific primers (AMACR: 5′-GAATCCGTATGCCCCGCTGAATCT-3′ and 5′-ACCCTTGCCAGTGCGTGTGC-3′; TBP: 5′-CACGAACCACGGCACTGATT-3′ and 5′-TTTTCTTGCTGCCAGTCTGGAC-3′), as described previously (16). A standard curve was generated by serial dilution of plasmids containing the specific amplicons assayed. AMACR mRNA copy number was normalized to TBP.

Western Blot Analysis. Protein (25 μg) was subjected to SDS-polyacrylamide (10%) gel electrophoresis, transferred to nitrocellulose (Amersham Pharmacia Biotech, Piscataway, NJ), probed with AMACR antiserum (7) at a 1:2000 dilution, and detected by enhanced chemiluminescence (Amersham Pharmacia Biotech) as described previously (16).

TMAs: Construction and Analysis. A total of 159 radical prostatectomy specimens were selected randomly out of a total of >400 cases performed between 1/1/2000 and 8/1/2001 at Johns Hopkins Hospital and used to construct TMAs as described previously (16). Areas representing the largest carcinoma present, as well as areas of normal appearing prostate epithelium, were circled on the glass slides. For each sample of tumor and normal, four tissue cores were taken for TMA construction. Although there is no universal method of sampling prostate cancer tissue for IHC studies, using either standard slides or TMAs, the histological features of these areas that we sampled generally reflect the final Gleason score for the case. Stained TMA slides were scanned using the BLISS imaging system as described by Manley *et al.* (17). Each array spot was then formed into a composite image and viewed and scored on a personal computer monitor as described (16). Data were then further summarized, and statistical analysis was performed using Stata 6.0 and SAS for Microsoft Windows.

Prostate Adenocarcinoma Tissue from Standard Slides. For a number of the cases, standard slides were used to assess overall percentage of positive cells in the tumors and normal tissues and also to further evaluate expression in HGPIN and BPH.

Metastatic Prostate Cancers. For metastatic carcinoma, standard slides from specimens (pelvic lymph node, soft tissue, and bone metastases) from patients with nonhormone refractory tumors were obtained from the archives of the Department of Pathology at The Johns Hopkins University. For hormone refractory cancer specimens, tissues were obtained as a single TMA from the University of Michigan Specialized Programs of Research Excellence core tissue facility.

IHC Staining. Staining for AMACR was carried out using the Envision+ kit (DAKO Corp., Carpinteria, CA). Briefly deparaffinized slides were hydrated and then placed in citrate buffer (pH 6.0) and steamed for 14 min. Endogenous peroxidase activity was quenched by incubation with DAKO peroxidase block for 5 min at room temperature. Slides were then washed and incubated with primary antibody (1:16,000 dilution of antiserum) overnight at 4 C. Secondary antirabbit antibody-coated polymer peroxidase complex was applied for 30 min at room temperature. Substrate/chromogen was applied and incubated for 5–10 min at room temperature. Slides were counterstained with hematoxylin. For double labeling of AMACR and p63, the anti-p63 mouse monoclonal antibody cocktail (1:100 dilution; Lab Vision Corp., Fremont, CA) was added after the anti-racemase antibody incubation and incubated for 45 min at room temperature. The secondary antirabbit and antimouse HRP conjugates were sequentially added, and the reaction was developed as above.

Scoring of IHC Staining. A scoring method was based on the fact that the specimens clearly showed a varying degree of staining intensity and percentage of cells staining. Therefore, a combined intensity and percentage positive scoring method was used (18). Strong intensity staining was scored as 3, moderate as 2, weak as 1, and negative as 0. For each intensity score, the percentage of cells with that score was estimated visually. A combined weighted score consisting of the sum of the percentage of cells staining at each intensity level was calculated for each sample, *e.g.*, a case with 70% strong staining, 10% moderate staining, and 20% weak staining would receive a score as follows: (70 × 3 + 10 × 2 + 20 × 1) = 250. The maximum score is 300.

Results

Analysis of Paired Samples of Normal and Cancerous Prostate Tissue mRNA. In the previous study, we used weighted gene and random permutation analysis to identify 210 genes with statistically different levels of mRNA expression between prostate cancer and BPH (3). Among these genes, AMACR maintained consistently low levels of expression in 9 of 9 BPH samples (signal intensities in the lowest quartile of all genes analyzed) but was overexpressed by an average of 5.7-fold in 13 of 16 cancer samples. We have since generated mRNA profiles of an additional 35 prostate samples, including 12 matched normal cancer pairs and 11 nonpaired cancer samples (data set in preparation for publication) by comparing each sample to a common reference (from BPH). To illustrate the expression pattern of AMACR in these samples in relation to other genes whose expression in prostate tissues has been well documented previously, we extracted expression ratios for AMACR (IMAGE clone ID: 133130), along with those for GSTP1 (IMAGE clone ID: 136235) and KRT8 (IMAGE clone ID: 897781). Reduced GSTP1 expression because of "CpG island" hypermethylation is found in >90% of prostate cancers (19), and KRT8 mRNA is expressed constitutively in the epithelial cells of normal and cancerous prostate tissues (20). As shown in Fig. 1, the majority (20 of 23) of the cancer samples demonstrated overexpression of AMACR mRNA compared with the BPH reference. When the cancer samples were compared with their matching normal samples, AMACR was overexpressed in the cancer samples in 9 of 12 pairs. Moderate overexpression compared with the reference (an average of 3-fold) was also observed in 3 of the 12 normal samples. Consistent with previous reports, GSTP1 expression was down-regulated in the majority of cancer samples compared with BPH reference or their matching normal samples. Expression of KRT8, an epithelial marker, showed little variation among all of the samples, indicating that the sample preparation was effective to enrich and balance the epithelial content in the samples analyzed.

A quantitative reverse transcription-PCR assay was used to estimate the relative difference in AMACR mRNA abundance in samples of normal and cancerous prostate tissue. Although extensive variability was observed, the copy number of AMACR mRNA in prostate cancer specimens was on average 8.8-fold higher than the value for normal prostate samples (average = 60.9, SD = 84.3, range 0.6–260.4, n = 8, for cancer samples compared with an average of 6.9,

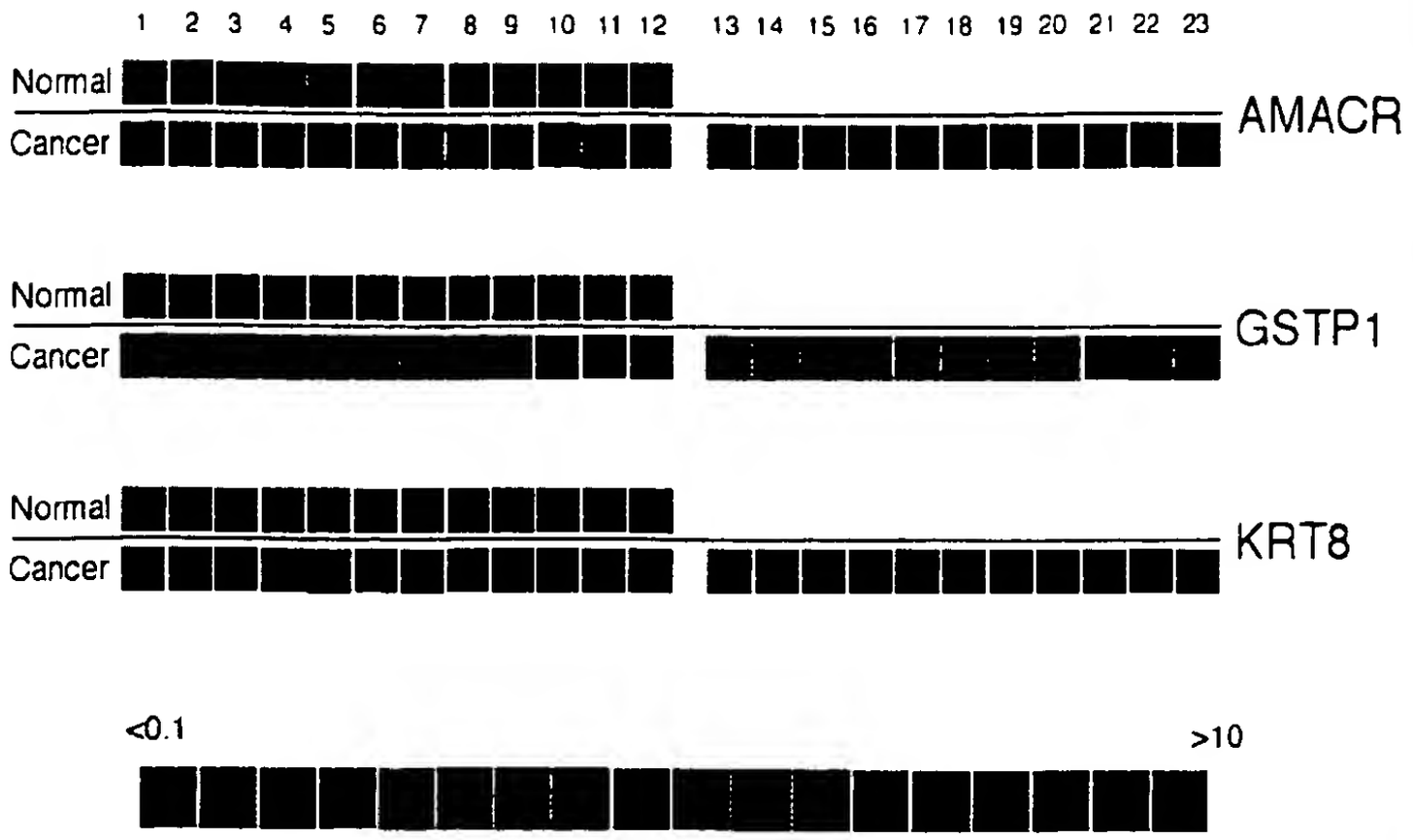

Fig. 1 Gene expression ratios for AMACR, GSTP1, and KRT8. Each *colored square* represents the relative mRNA abundance ratio of sample:reference1 in each sample compared with the common BPH reference. Each of the 12 normal samples (*1–12*) was paired with its matching cancer sample below the *black line* and the other 11 nonpaired cancer samples (*13–23*) positioned at the *right side below* the *line*. The measured expression ratios for each gene were presented graphically as *colored squares*, with the *green squares* representing higher expression in sample compared with the reference (BPH), the *red squares* meaning lower expression in sample than reference (BPH), and *black squares* indicating a ratio of ~1. Color intensities are scaled according to the ratio (sample:reference1) as shown at the *bottom*, with the brightest color having a ratio of >10 (*green*) or <0.1 (*red*).

SD = 9.9, range 0.51–32.9, n = 8, for normal) when normalized against copies of TBP. Similarly, the median value for the cancer samples was 7.8-fold higher than for the normal samples.

Western Blot Analysis of AMACR Protein. Expression of AMACR protein was examined using an antiserum demonstrated previously to be specific for this antigen (7). Western blot detection of AMACR in liver, as a positive control (7), and a series of prostate tissue samples is shown in Fig. 2. Although a M_r 47,000 band corresponding to AMACR protein was readily detected in liver and each of the prostate cancer specimens, little or no reactivity was observed in the corresponding normal specimens or any of the BPH samples assayed.

IHC Analysis of Radical Prostatectomy Specimens Using Standard Slides. To obtain the pattern of staining of AMACR in prostate tissues, we examined clinically localized prostate adenocarcinoma specimens using representative standard slides containing normal epithelium, carcinoma, and HGPIN. We used a scoring method that accounts for both the intensity of staining and the percentage of cells staining. In general, normal prostate epithelium was either negative or weakly positive (n = 14 areas from 12 patients, median score = 15, mean score = 19, SD = 22; Fig. 3). Prostate stroma, inflammatory cells, endothelial cells, and nerves were uniformly negative (Fig. 3). Strikingly, adenocarcinomas showed highly intense staining in the majority of tumor cells in most cases (n = 19 carcinoma lesions from 14 patients, median = 290, mean = 277, SD = 30; Fig. 3). The staining was uniformly cytoplasmic and was typically found in small punctate, microbody structures, consistent with the previously reported localization of AMACR predominantly to peroxisomes and mitochondria (7). Staining in HGPIN was also generally positive, although the staining was more variable and often less intense than adjacent carcinoma (Fig. 3, *C* and *D*; n = 10 areas from 6 patients, median 170, mean 179, SD = 63). Staining in atrophic areas was generally negative or positive in a small percentage of cells (n = 5 areas from 3 patients, median = 30, mean = 30, SD = 22). Staining in BPH was generally negative or focally, weakly positive (n = 6 areas from 4 patients, median = 20, mean = 20, SD = 8.9).

IHC Analysis of Radical Prostatectomy Specimens Using TMAs. To survey many tumor and normal specimens, four high-density TMAs, designed to contain samples of clinically localized prostate cancer and matched normal appearing epithelium from 159 patients, were stained. The median age of patients in these TMAs was 58 (range 40–73), and the median prostate-specific antigen was 6.4 (mean 7.5, SD 4.6, range 1.2–38). Array spots (1578) were imaged. The majority of array spots (79%) contained tissue that was readily readable. Of the usable array spot images, 209 were control normal (nonprostatic) tissues. Of the array spots scored for AMACR staining, 417 spots contained adenocarcinoma (334 Gleason pattern 3, 62 Gleason pattern 4, 15 Gleason pattern 5, 4 Gleason pattern 2, 1 ductal adenocarcinoma, 1 pseudo-hyperplastic carcinoma) from 142 patients, 442 spots contained normal prostate epithelium from 144 patients of the patients, and 27 spots contained HGPIN from 23 patients. Other spots consisted of prostate stroma only, atrophy, or were difficult to interpret.

For normal prostate and adenocarcinoma, between 2.5 and 3 TMA spots (duplicate spots) were scored from each patient. For statistical analyses, the mean of the individual spot scores were computed so that for each tissue type from each patient, there was a single IHC score for comparison. Using these mean scores, the AMACR IHC scores for both carcinoma and HGPIN were highly statistically significantly different from normal (Fig. 4, carcinoma *versus* normal, $P < 0.0001$;

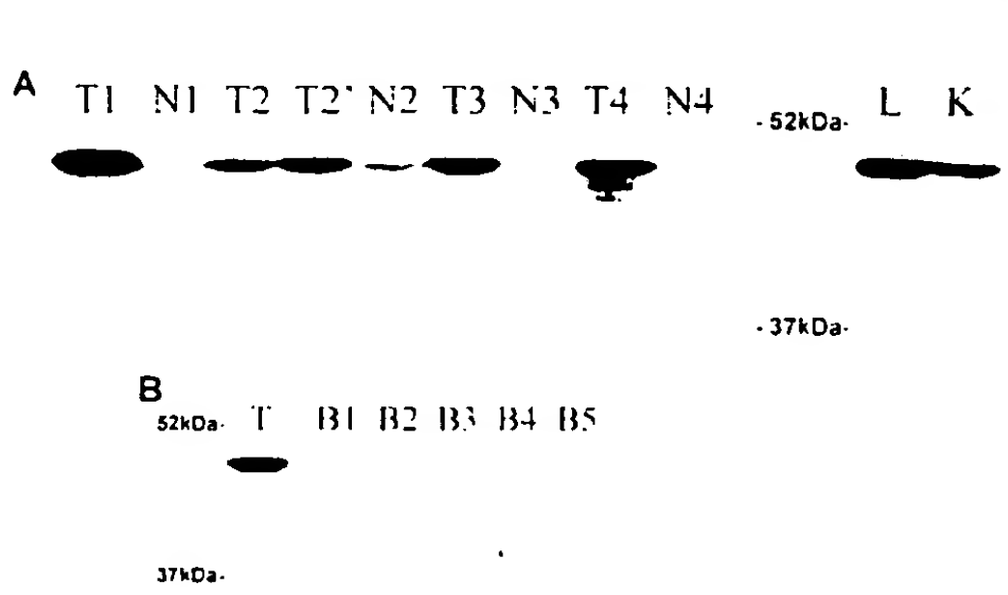

Fig. 2. Western blot analysis of AMACR protein. Total tissue protein (25 μg) was analyzed using anti-AMACR antiserum. In *A*, pairs of tumor and corresponding normal tissue from 4 patients are designated by *T1, T2, T3, T4*, and *N1, N2, N3, N4*, respectively. For the second patient, there were two apparently independent tumors sampled (*T2* and *T2'*). *L*, liver and *K*, kidney. In *B*, five different samples of BPH tissue (*B1–B5*) were analyzed together with a single sample of prostate cancer (*T*). The position of prestained molecular weight markers (ovalbumin at M_r 52,000 and carbonic anhydrase at M_r 37,000) is marked.

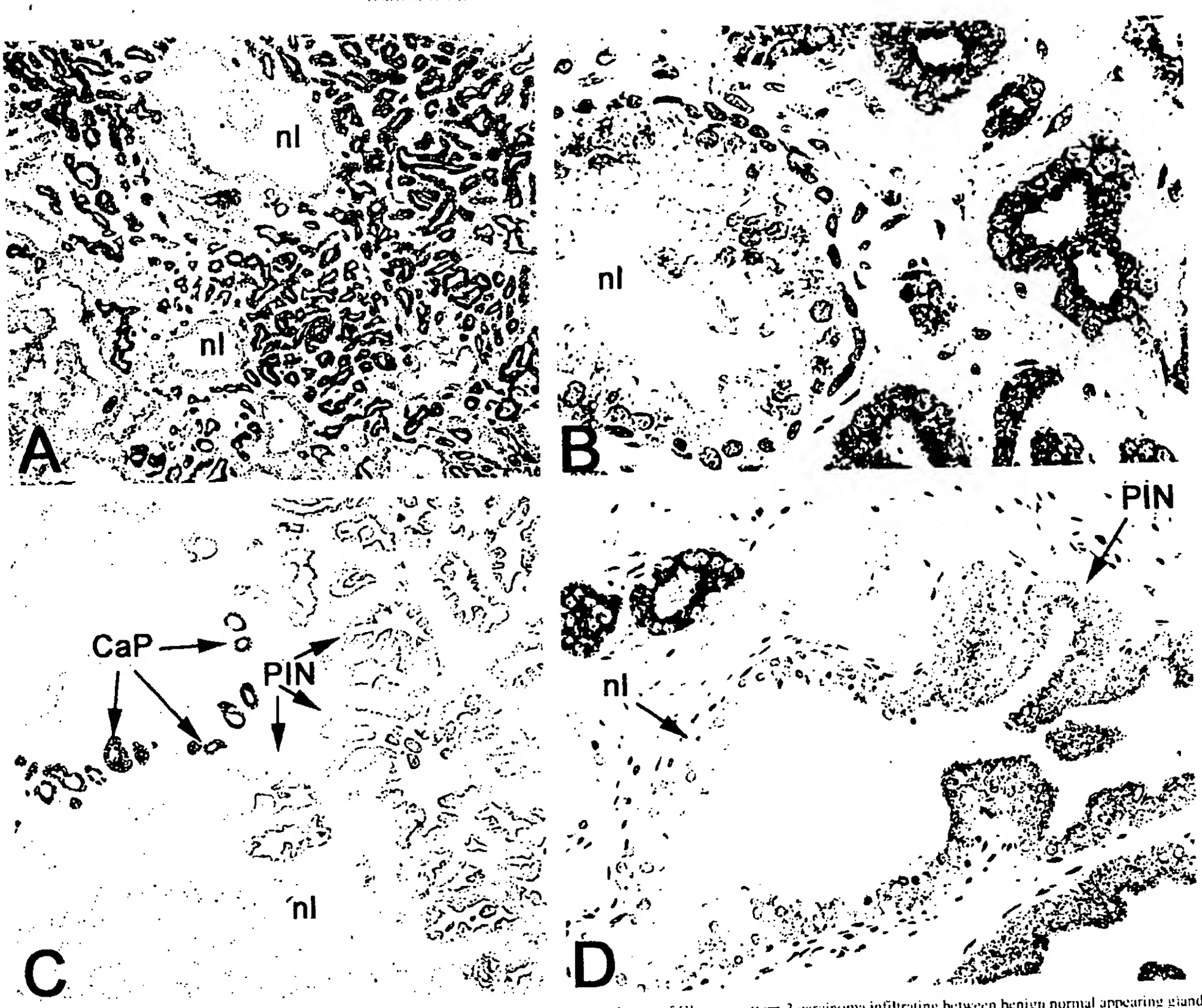

Fig. 3. IHC localization of AMACR in prostate tissue using standard slides. *A*, low-power image of Gleason pattern 3 carcinoma infiltrating between benign normal appearing glands (*nl*) showing homogeneous strong staining (×40). *B*, higher power view of representative region from *A* showing punctate cytoplasmic staining (×400). *C*, another case showing high-intensity staining in carcinoma (*CaP*), intermediate intensity staining in HGPIN (*PIN*), and no staining in normal (*nl*; ×100). *D*, higher power view of *C* showing strong staining in carcinoma gland (*top left*) and staining in acinus in that is involved partially with HGPIN with staining only in atypical HGPIN cells (*PIN*). Note negative staining in the normal appearing acinus just to the *right* of the carcinoma (×200).

HGPIN *versus* normal, $P < 0.001$, Wilcoxon's rank-sum test). The score for HGPIN was significantly less than the score for carcinoma ($P = 0.0002$, Wilcoxon's rank-sum test). If a cutoff of ≥100 is used for positive staining, then 95.6% of carcinomas were positive, whereas 3.5% (5 of 144) of normal epithelium was positive. Using a score of ≥150 for strong positive staining, then 88% of the carcinomas would be considered strong, *versus* none of the normal epithelium. The carcinomas (52%) had a median score of 300 (the maximum).

The distribution of AMACR mean IHC scores for carcinoma (mean for all of the spots for each patient) stratified by Gleason score and pathological stage is shown in Table 1. There was no relation between AMACR IHC score and Gleason grade, pathological stage, patient age, or preoperative serum prostate-specific antigen (Kruskal-Wallis, all Ps > 0.05). As expected, Gleason score at radical prostatectomy was associated strongly with higher stage disease (Spearman's rank correlation coefficient = 0.42, $n = 142$, $P < 0.0001$; Table 1).

IHC Analysis of Prostate Cancer Metastases. Staining in metastatic prostate cancers from nonhormone refractory disease ($n = 32$ sites from 32 patients: 8 bone, 21 pelvic lymph node, 2 soft tissue, and 1 lung) showed staining in the majority of cases (data not shown). The median score in nontreated metastatic cases was 240 (mean 204, SD 97). By the criteria stated above for positivity, 81% (26 of 32) were positive, and 62.5% were strongly positive (20 of 32). In hormone refractory metastatic prostate ($n = 25$ sites from 14 patients), the median score was 215 (mean = 206, SD = 84). Thus, 93% (13 of 14) of hormone refractory metastatic cancers were positive, and 71.4% were strongly positive.

AMACR Protein Expression in Other Normal Tissues. To examine the overall tissue distribution of AMACR protein expression, staining was performed using a TMA containing a wide variety of human tissues. Staining was strongly positive in virtually all hepatocytes, proximal kidney tubules, and glomerular epithelial cells of the kidney (data not shown). Moderate staining was found in the acinar cells and some ductal cells of major salivary glands. Weak staining was found in distal tubules and collecting ducts of the kidney. Other tissues showed weak and heterogeneous expression, including the following: (*a*) neurons in the central nervous system; (*b*) absorptive

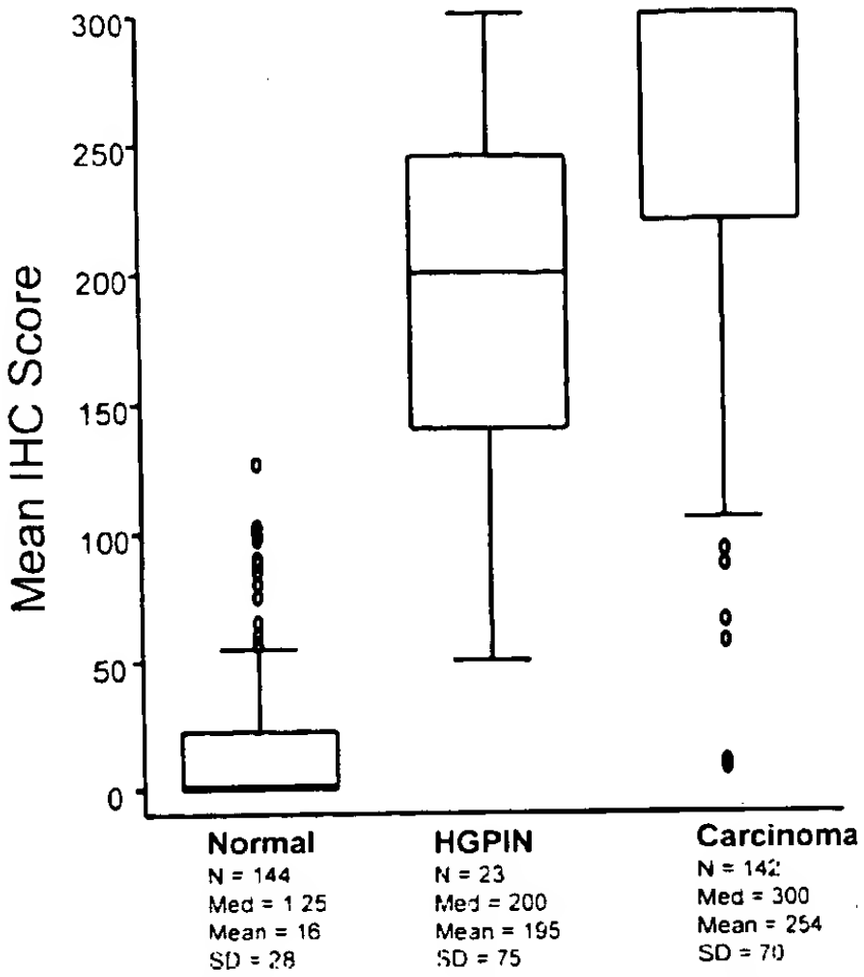

Fig. 4. Relative expression of AMACR by IHC score in normal, HGPIN, and carcinoma by scoring of TMA spots. *Box* shows 25th-75th percentiles, as well as median (*center line*). *Whiskers* show 5th and 95th percentiles, and *ovals* represent outliers.

and paneth cells of the small bowel; (*c*) absorptive cells of the large bowel; and (*d*) Sertoli cells of the testis. Staining was not detectable in the urinary bladder, ovary, endometrium, fallopian tube, uterine cervix, breast, lung, skin, tonsil, lymph nodes, thymus, spleen, laryngeal epithelium, minor salivary glands, pancreas, gall bladder, thyroid, stomach, esophagus, skeletal muscle, or smooth muscle.

Combination of AMACR Staining with p63 Facilitates Detection of Prostate Cancer Cells. The intense staining in tumors with weak staining in normal suggested that AMACR IHC staining might be useful as an adjunct to the diagnosis of prostate cancer on needle biopsy or other clinical specimens. To be useful as a potential diagnostic marker for prostate cancer, carcinoma lesions need to be separated reliably from lesions of HGPIN and other potential mimicars of prostate cancer, such as adenosis and atrophy, as well as from normal prostate tissue. On small needle biopsy samples, this distinction can sometimes be problematic (21). One very useful tool has been IHC staining for basal cell-specific cytokeratins, typically using the 34BE12 monoclonal antibody (22). Because basal cells, which are absent in the vast majority of prostatic adenocarcinomas, are present in normal glands, benign mimicars, such as atrophy and adenosis, and in HGPIN, staining for basal cell cytokeratins is often used in prostate cancer needle biopsies (22). More recently, the p63 protein has been found to be localized to the nuclei of basal cells in prostate epithelium with a basal cell-specific staining pattern nearly identical to 34BE12 (13, 14). Because the staining for p63 is strictly nuclear and is negative in the vast majority of carcinomas, and the staining for AMCAR is cytoplasmic and only strong in carcinoma or HGPIN, we combined the staining for these two markers as a cocktail. There was strong staining of the nuclei of basal cells in benign glands, whereas carcinoma showed no nuclear staining but strong cytoplasmic staining, with no decrease as compared with AMCAR staining alone ($n = 20$, Fig. 5, *A* and *B*). HGPIN showed variable punctate, cytoplasmic AMCAR staining, but strong and homogeneous nuclear staining in basal cells, thus allowing one to reliably distinguish HGPIN from carcinoma (Fig. 5, *C* and *D*). At times, HGPIN appeared to contain buds of epithelium, pinching off into the underlying stroma (23). An example of this phenomenon is shown in Fig. 5*D* (*arrow*), where a single acinus is projecting from an acinus containing HGPIN. The projecting acinus shows very sparse p63 basal cell staining, consistent with early invasion. The surrounding carcinoma cells show complete loss of p63 staining, yet they contain AMACR cytoplasmic staining.

Discussion

In this study, we demonstrate that AMACR is overexpressed in the majority of prostate cancers and cancer cells within these tumors. Using many cases with diverse pathologic characteristics, we find that overexpression at the protein level is very tightly linked to prostate cancer and occurs in virtually all grades and stages and in both hormone refractory and untreated cases. Over 95% of prostate cancers analyzed stained positively for AMACR, whereas <4% of histologically normal prostate epithelium was positive. Using a more stringent scoring scheme, these values are 88 and 0% for cancers and normal cells that are positive, respectively. Because there appears to be such a tight link between overexpression and the histological prostate cancer phenotype, these finding have implications for the pathogenesis, diagnosis, imaging, and treatment of prostate cancer.

In terms of early diagnosis on prostate needle biopsy, whereas basal cell-specific keratin is a useful aid for diagnosis, this marker is lost in prostate cancer. Because there can be artifactual loss of IHC staining, at times, this staining is uninformative and/or misleading. To date, few, if any, validated markers of prostate cancer have been identified that are overexpressed consistently as detected by IHC staining such that they might be of diagnostic use in a large percentage of cases. We now show that AMACR may provide the first example of such a marker. Because AMACR staining can occur intensely in HGPIN, we combined staining using AMACR with staining for p63. Because these markers, when present, are invariably cytoplasmic and nuclear, respectively, there is no need for the use of two color staining. p63 staining has been shown to be comparable with the basal cell-specific keratin 34βE12 monoclonal antibody but has the advantage in this case of a more clearly discernible nuclear location (13, 14). Thus, AMACR is a new positive stain that complements the traditional negative stain to enhance prostate cancer diagnosis. To determine the usefulness of AMACR in diagnostic pathology practice, we have begun to use clinical needle biopsies in prospective studies comparing AMACR, AMACR/p63, and basal cell-specific cytokeratins. Additionally, this marker combination could be of use for nonpathologist researchers studying prostate cancer specimens to enhance diagnostic certainty.

The enzyme encoded by this gene plays a critical role in the metabolism of fatty acid molecules, specifically by peroxisomal β

Table 1 *AMACR IHC score from TMA analysis for 142 patients stratified by Gleason grade and pathological stage at radical prostatectomy*

Grade		Stage			
		T2	T3A	T3B	N1
5–6	n	65	12	2	0
	Median	300	268	258	Na[a]
	Mean	255	218	258	Na
	SD	68	100	60	Na
7	n	24	14	5	1
	Median	293	300	243	300
	Mean	238	263	254	300
	SD	83	60	43	0
8–9	n	5	10	3	1
	Median	300	300	283	300
	Mean	297	274	273	300
	SD	8	58	34	0

[a] Na, not applicable.

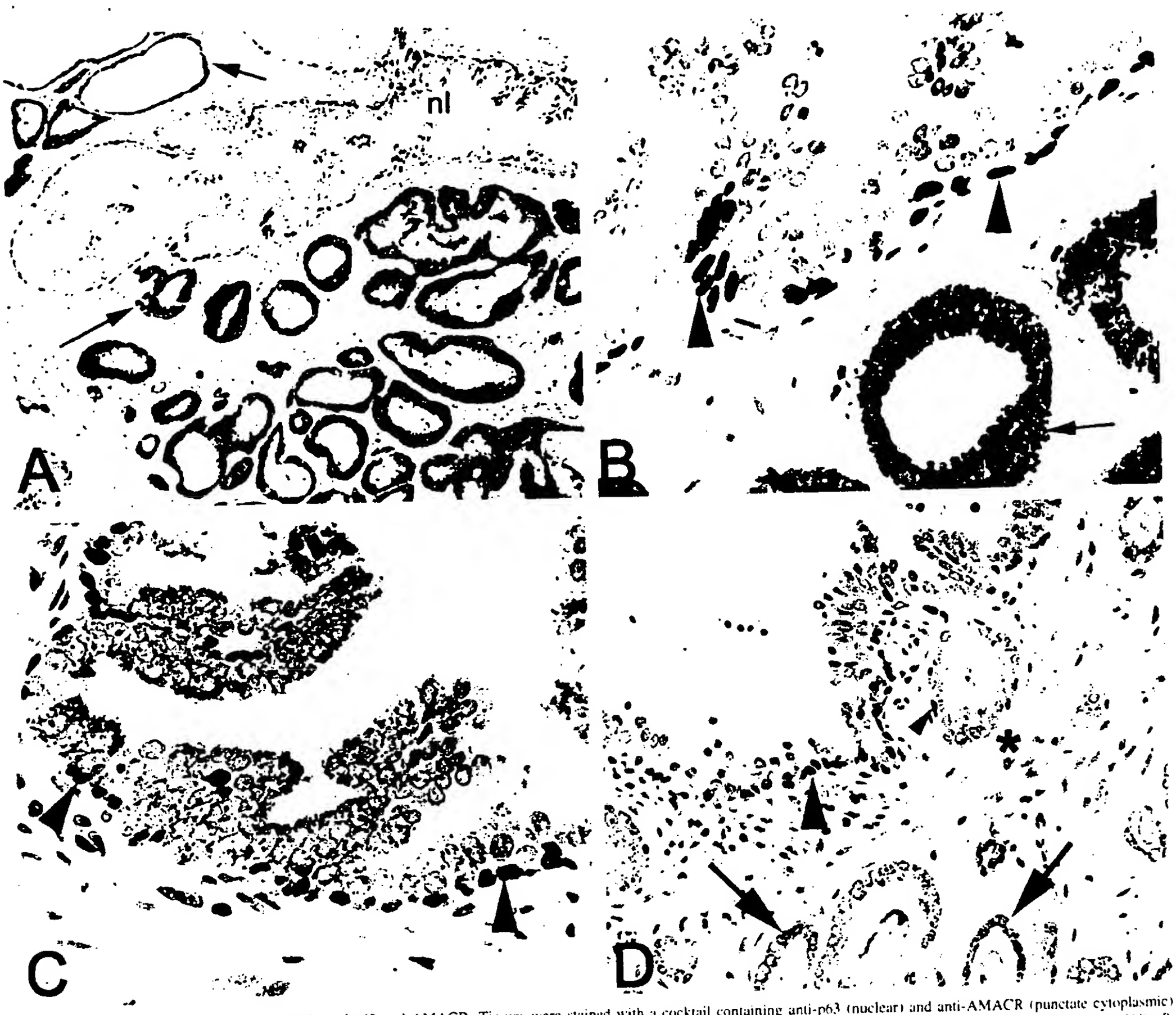

Fig. 5. Simultaneous single color localization of p63 and AMACR. Tissues were stained with a cocktail containing anti-p63 (nuclear) and anti-AMACR (punctate cytoplasmic) antibodies, and staining was localized simultaneously. In *A*, nuclear basal cell staining (p63) is apparent in normal appearing acinus. *Arrows*, infiltrating carcinoma cells (×100). *B*, higher power view of *A*. *Arrowheads*, nuclear basal cells staining (p63); *arrow*, carcinoma (×400). *C*, HGPIN showing moderate to weak punctate cytoplasmic AMACR staining and strong positive staining p63 staining of basal cells (×400). *D*, HGPIN (top gland) with weak cytoplasmic AMACR staining and nuclear basal cells staining (p63). Note small acinus apparently budding off (*) of HGPIN gland showing very sparse basal cell nuclear staining (*arrowhead*). *Below*, there is moderate to weak intensity cytoplasmic staining in infiltrating carcinoma cells that lack p63 nuclear staining.

oxidation (10). Branched chain fatty acids, which originate almost entirely from the diet, contain methyl groups in the R position, whereas the enzymes of the β oxidation pathway can only transform substrates having the S configuration (10). The enzyme AMACR catalyzes this interconversion. One implication of the up-regulation of AMACR is that prostate cancer cells may have a consistently greater capacity to metabolize dietary branched chain fatty acids than would their normal counterparts. Although the contribution of this up-regulation to prostate carcinogenesis, if any, is unclear at present, two interesting aspects of this pathway may be relevant: (*a*) the first step of the pathway in β oxidation of branched chain fatty acids is an oxidation step catalyzed by acyl-CoA oxidases, with the products being oxidized substrate and hydrogen peroxide (10). Experimental overexpression of acyl-CoA oxidase can transform cells (12), and the increase in oxidative stress because of the production of hydrogen peroxide by this pathway has been proposed to play a role in the transformation process (11). Indeed, the potent carcinogenic activity of peroxisome proliferators in animal models is thought to be mediated, at least in part, by up-regulation of the peroxide-producing enzymes of the peroxisome (24); and (*b*) the primary branched chain fatty acid whose metabolism is critically dependent on the action of AMACR is phytanic acid, derived from phytol, a breakdown product of chlorophyll in ruminants. This fatty acid is found primarily in cow's milk, and diary products derived there from, as well as beef but not meat from chickens or some fish (25). An interesting question is whether the increased risk for prostate cancer conferred by consumption of dairy products and/or red meat (9) is related to the up-regulation of this enzyme and its associated pathway in the early stages of prostate carcinogenesis, *e.g.*, in PIN.

Molecular imaging promises the ability to identify individual cells or groups of cells expressing specific proteins or enzymatic activity in real time in living patients (see Louie *et al.*; Ref. 26) The ability to

image AMACR protein or enzymatic activity would likely provide significant value in localization of primary prostate cancer within the prostate. A first application of this would be to help direct the location of needle biopsy sites in the prostate and possibly to assess the extent of cancer within the prostate. In addition, the ability to image AMACR systemically would provide value for detection of metastatic prostate cancer in organs other than the liver and kidney.

The prostate glands of U.S. men are biopsied ~1 million times a year, leading to a positive diagnoses of ~180,000 new prostate cancer cases annually. Estimates of equivocal or ambiguous biopsy evaluations that are suspicious for cancer range from 0.3 to 24% (21), resulting in tens of thousands of repeat biopsies. Clearly, markers, which can assist in the accurate evaluation of prostate needle biopsies, are needed urgently. Because of its consistency and robustness, AMACR appears to fit the criteria for such a marker and as such may become routinely used in the pathological diagnosis of prostate cancer. In addition, the biological function of this marker provides exciting new information regarding the etiology of prostate cancer and provides a novel target for prevention and therapeutics. Whether this marker is pathogenic, a potential drug, or *in vivo* imaging target is subject to ongoing studies.

While our manuscript was in preparation, Jiang *et al.* (27) reported overexpression of AMACR by IHC, using a different antibody, in the majority of primary prostate cancers. Although this report did not examine AMACR expression in other tissues, or combine AMACR staining with p63 or evaluate metastatic prostate cancers, the fact that two separate groups simultaneously observe marked overexpression of this protein in primary prostate cancer is a remarkable finding that bolsters the overall importance of AMACR as a new prostate cancer marker.

Acknowledgments

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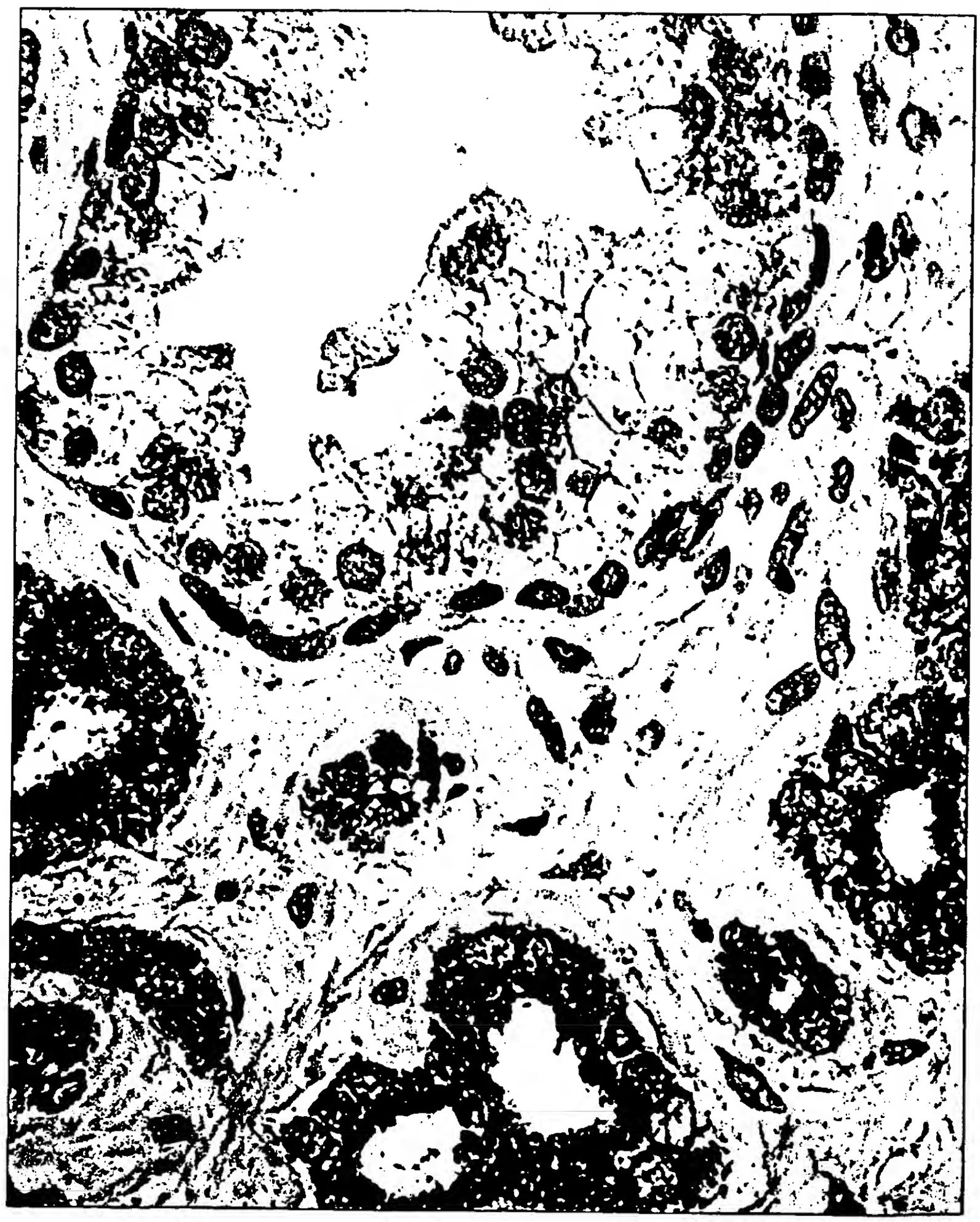

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Exhibit D

The American Journal of Surgical Pathology 26(9): 1169–1174, 2002

P504S/α-Methylacyl-CoA Racemase

A Useful Marker for Diagnosis of Small Foci of Prostatic Carcinoma on Needle Biopsy

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Establishing a definitive diagnosis of malignancy in prostate needle biopsies with very small foci of adenocarcinoma is a major diagnostic challenge for surgical pathologists. A positive diagnostic marker specific for prostatic adenocarcinoma may enhance our ability to detect limited prostate cancer and reduce errors in diagnosis. P504S, also known as α-methylacyl-CoA racemase, recently identified by cDNA subtraction and microarray technology, might serve as such a specific marker because it has been demonstrated to be highly expressed in prostatic adenocarcinoma, but not in benign prostatic glands. However, whether small foci of carcinoma can be reliably detected by this marker is a crucial question for its clinical application. The aim of this study was to assess the utility of P504S immunohistochemistry in detecting small amounts of prostate cancer in prostate needle biopsies. A total of 142 prostate needle biopsies, including 73 cases with a small focus of prostatic adenocarcinoma (≤1 mm) and 69 benign prostates, were examined by using immunohistochemistry for P504S and high molecular weight cytokeratin (34βE12). P504S immunoreactivity was found in 69 of 73 cases (94.5%) of carcinoma but not in any benign prostates (0 of 69) or benign glands adjacent to malignant glands. The 34βE12 immunostaining confirmed the absence of basal cells in the focus of carcinoma in all 73 cases. The high specificity and sensitivity of P504S in the detection of minimal prostatic adenocarcinoma indicated its potential diagnostic value in clinical practice. Using a combination of P504S and 34βE12 can help the diagnosis of limited prostatic adenocarcinoma on needle biopsy.

Key Words: P504S—α-Methylacyl-CoA racemase—Limited prostate carcinoma.

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In recent years efforts to improve the early detection of prostate cancer by use of serum prostate-specific antigen testing have resulted in increased numbers of prostate needle biopsies, which show small foci of prostatic adenocarcinoma.[3–6] The diagnosis of these small foci of prostate cancer on needle biopsy is one of the major diagnostic challenges in surgical pathology.[4,5] Underdiagnosis of a small focus prostatic adenocarcinoma or the overdiagnosis of a benign lesion mimicking cancer on prostate needle biopsy is not uncommon[15] and can cause unfortunate consequences for patients and is a potential liability for pathologists. It will be of great value to have a sensitive and specific marker for detection of prostatic adenocarcinoma in clinical practice.

P504S, a cytoplasmic protein also known as α-methylacyl-CoA racemase, was recently identified by cDNA library subtraction in conjunction with high throughput microarray analysis of prostatic adenocarcinoma.[17] P504S mRNA is significantly elevated in prostate cancer compared with benign prostate. Recently, it was reported that high expression of P504S protein was found in prostatic adenocarcinoma but not in benign prostatic tissue by immunohistochemical staining.[11] These findings suggest that P504S has the potential to be a useful diagnostic marker for prostate cancer in clinical practice.[11] How useful P504S will be as a diagnostic marker will depend on its ability to distinguish small foci of prostatic carcinoma from benign glands on needle biopsy specimens. To address this critical question, we investigated the feasibility of using P504S immunohistochemistry in the detection of small foci of prostatic adenocarcinoma in prostate needle biopsies.

MATERIALS AND METHODS

Cases

A total of 142 prostate needle biopsies, including 73 cases of small foci of prostatic adenocarcinoma and 69

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benign prostates, were obtained from the surgical pathology files at the University of Massachusetts Medical Center, the University of Chicago Hospitals, or Massachusetts General Hospital. A small focus of prostatic adenocarcinomas was defined as a tumor focus of ≤1 mm in diameter, which was present as a single focus either in the entire biopsy specimen (N = 44) or in one side of the prostate in addition to carcinoma present on the contralateral side (N = 29). All tissue was fixed in 10% neutral buffered formalin and paraffin embedded.

Immunohistochemistry

Formalin-fixed, paraffin-embedded tissue blocks were cut into 5-μm sections, transferred to glass slides, and treated with 0.1 mol/L citrate buffer, pH 6.0, in an 800-W microwave oven for 15 minutes for antigen retrieval before immunostaining. The slides were stained on a TechMate 1000 (Ventana Medical Systems, Tucson, AZ, USA) automated immunostainer using an avidin/biotin complex staining procedure. The sections were incubated with a rabbit MAb to P504S at a 0.5-μg/mL dilution or MAb 34βE12 (DAKO, Carpinteria, CA, USA) to high molecular weight cytokeratin at a dilution of 1:50 for 45 minutes followed by brief buffer washes. Then sections were incubated with a cocktail of biotinylated anti-rabbit IgG and anti-mouse IgG/IgM (Ventana) for 30 minutes. The sections were then washed, incubated in avidin/peroxidase complex (Ventana) for 30 minutes, washed, and then reacted with diaminobenzidine and hydrogen peroxide to visualize the end product. The sections were counterstained with hematoxylin.

To confirm the specificity of the anti-P504S staining, the anti-P504S antibody was preincubated with eightfold excess of P504S recombinant protein. The preabsorption procedure was found to block immunostaining in prostate tissues, indicating that the staining observed was specific for P504S. In addition, a duplicate set of slides was also stained with an irrelevant rabbit MAb, anti Von Willebrand factor, as a negative control. The irrelevant MAb did not stain benign or malignant prostatic glands.

Morphologic Evaluation

The diagnosis of prostate cancer was established from examination of hematoxylin and eosin-stained sections by at least two pathologists and further confirmed by the lack of basal cells as shown by 34βE12 immunostaining. The Gleason scores were 3 + 3 (6) (N = 70), 4 + 3 (7) (N = 1), and 4 + 4 (8) (N = 2). Positive P504S staining was defined as continuous dark cytoplasmic staining or apical granular staining patterns in the epithelial cells, which can be easily observed at low power magnification (<100×). Scant fine granular background staining of epithelial and stromal cells, which cannot be seen at low-power magnification (≤100×), was considered negative.

RESULTS

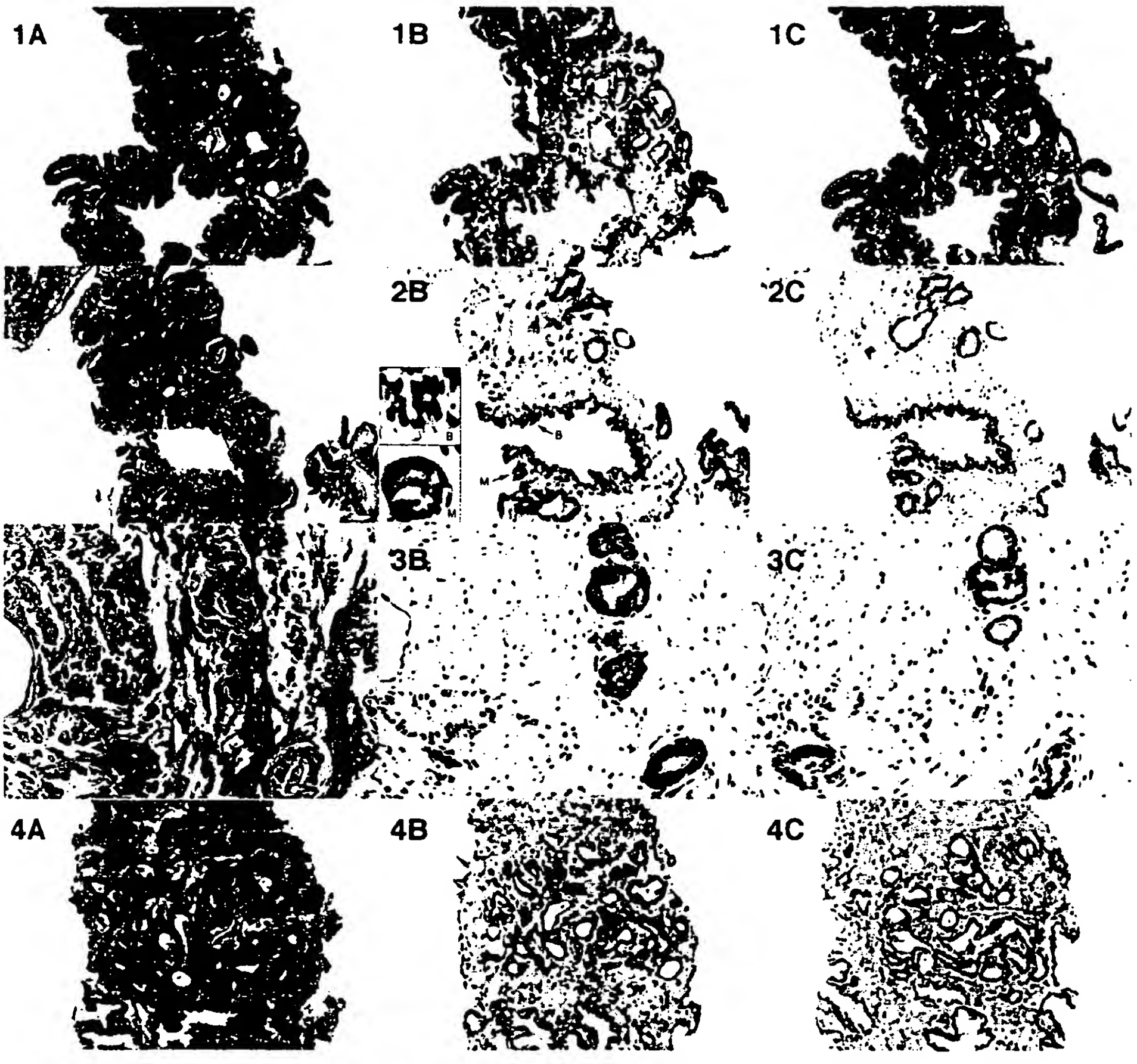
Sixty-nine of 73 (94.5%) cases of small foci of prostatic adenocarcinoma (Figs. 1A, 2A, 3A, and 4A, hematoxylin and eosin stain) expressed P504S (Figs. 1B, 2B, 3B, and 4B), whereas all the malignant glands were negative for basal cell staining (Figs. 1C, 2C, 3C, and 4C). The P504S positivity was detected in >75% malignant glands in 63 of 69 cases, 51–75% of malignant glands in three of 69 cases, and 25–50% of malignant glands in three of 69 cases. A case of prostatic carcinoma with atrophic changes expressed P504S (Fig. 4B) and did not stain with basal cell marker 34βE12 (Fig. 4C). Benign glands adjacent to the malignant glands were negative for P504S and positive for 34βE12. There was no difference in the staining pattern of the cases with single foci of cancer in the entire biopsy and the cases with one tumor focus on one side of the needle biopsy. Three cases with Gleason score 6 and one with Gleason score 8 were negative for P504S. One of negative P504S cases (Gleason score 6) was foamy gland cancer.

In contrast, all benign prostatic biopsies (Figs. 5A, 6A, 7A, and 8A, hematoxylin and eosin stain) were negative for P504S (Figs. 5B, 6B, 7B, and 8B) but positive for 34βE12 (Figs. 5C, 6C, 7C, and 8C). Expression of P504S was not detected in small-crowed benign glands with atrophic changes (Figs. 5B and 6B), inflammation (Fig. 7B), or prominent basal cells (Fig. 8B), whereas all of them were positive for 34βE12 (Figs. 5C, 6C, 7C, and 8C). The results are summarized in Table 1.

DISCUSSION

Prostate cancer is the most common malignancy excluding skin cancer in American men.[12] Needle biopsy is the most commonly used procedure for the definitive diagnosis of prostate cancer. Each year millions of prostate needle biopsies are performed around the world. The diagnosis of prostate cancer based by morphologic examination is usually straightforward if substantial tumor is present in the prostate needle biopsy but can be very difficult if there are only a few malignant or atypical glands. With increasing efforts to detect prostate cancer early by mass screening of men, there have been an increasing number of small foci of cancer encountered on prostate needle biopsies.[3-6]

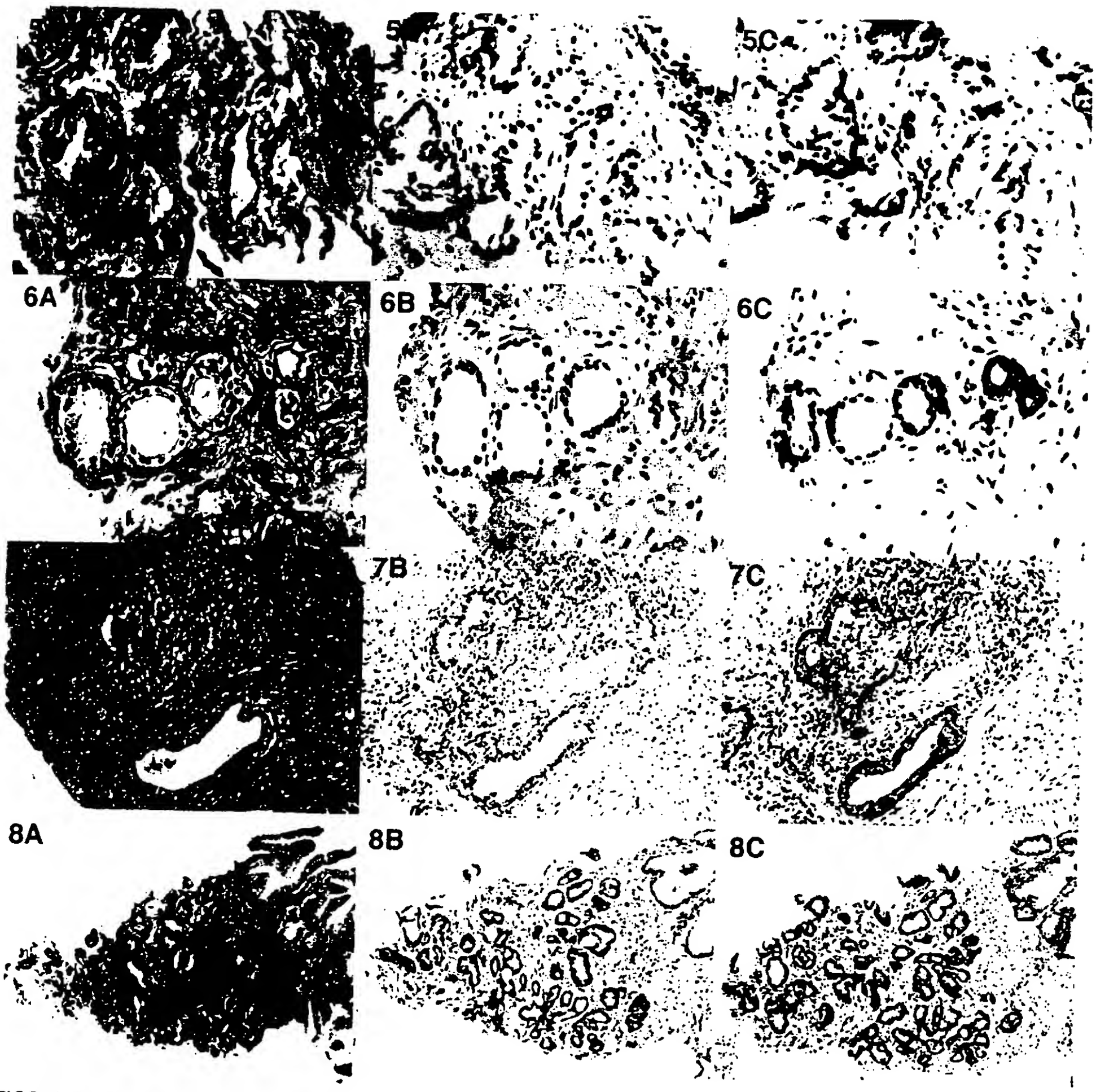
In this study we have demonstrated that P504S immunostaining detects small foci of prostatic adenocarcinoma on needle biopsy with 94.5% of sensitivity and



FIGS. 1–4. Comparison of hematoxylin and eosin **(A)**, P504S **(B)**, and 34βE12 **(C)** staining in small focal prostatic carcinoma from different cases of the prostate biopsies. **(A)** Several small clusters of malignant glands that are <1 mm but with architectural and cytologic atypia (**1**, **2**, and **3**), and small focal carcinoma with atrophic appearance (**4**). Serial sections of **B** (P504S staining) and **C** (34βE12 staining) show that malignant glands but not adjacent benign glands were positive for P504S whereas benign glands but not malignant glands were positive for 34βE12. The fine granular background P504S staining in benign epithelial cells (**2B**, arrow B, inset B), defined as negative staining, can be easily distinguished from the positive staining in malignant epithelia (**2B**, arrow M, inset M).

high specificity. The results indicate that P504S is an excellent marker for the pathologic diagnosis of prostatic adenocarcinoma even when the tumor volume is very limited in needle biopsies. Two independent groups of investigators, Rubin et al. at the University of Michigan and Luo et al. at Johns Hopkins University recently also reported that α-methylacyl-CoA racemase (P504S) was identified a prostate cancer marker by microarray technology.[13,14] Using immunohistochemical analysis with a polyclonal antibody, they confirmed that P504S/α-methylacyl-CoA racemase reactivity was detected in >95% of cases of prostate cancer. These studies support our findings and demonstrate that P504S is an important new marker for prostate cancer.

Several factors contribute to the difficulty in diagnosis of limited prostate cancer on needle biopsy. First, the malignant cells can be limited to a few glands that may be easily overlooked. Second, there is no single histologic feature specific and sufficient for the diagnosis of prostate cancer. The diagnosis is based on the com-



FIGS. 5–8. Comparison of hematoxylin and eosin **(A)**, P504S **(B)**, and 34βE12 **(C)** staining in benign glands from different cases of the prostate biopsies. **(A)** A small cluster of benign glands with atrophic appearance **(5)**, partial atrophy **(6)**, inflammatory glands **(7)**, and a cluster of small glands with basal cell proliferation **(8)**. Serial sections of **B** (P504S staining) and **C** (34βE12 staining) show that benign glands were negative for P504S but positive for 34βE12.

bination of architectural, cytologic, and extracellular material change.[4] Third, many benign prostatic conditions such as small-crowed glands, atrophy, inflammatory atypia, and basal cell hyperplasia can mimic prostate cancer histologically.[7] Fourth, the consequences associated with incorrect diagnosis can be serious, such as unnecessary prostatectomy or radiation associated with adverse complications resulting from a false-positive diagnosis and delay of effective treatment resulting from a false-negative diagnosis. Finally, because of sampling variations, a small focus of prostate cancer on biopsy may not necessarily represent a tumor of insignificant volume,[2,6,10] or the tumor may not be sampled on the rebiopsy. Therefore, it is important to make a definitive diagnosis using limited material if possible

To increase our diagnostic accuracy, it would be advantageous to have a biochemical marker to confirm the

TABLE 1. *Expression of P504S in minimal small foci of prostatic adenocarcinomas and benign prostates on needle biopsies*

Condition	No. of cases	Positive	Negative
Minimal prostatic adenocarcinoma	73	69 (94.5%)	4 (5.5%)
Benign prostates	69	0	69 (100%)
Benign glands adjacent to cancer	73	0	73 (100%)

diagnosis of prostate cancer on needle biopsy. High molecular weight cytokeratin (34βE12) has been demonstrated to be a negative marker that can aid in diagnosis of prostate carcinoma.[1,8,9,16] The positive 34βE12 staining in prostatic basal cells may render a definitive diagnosis of benign glands. However, the negative tumor marker has its limitation. First, it is helpful only if an abnormal focus is identified. If one fails to recognize a minute focus of cancer or a focus suspicious for cancer, a 34βE12 staining will unlikely be ordered to provide any help for diagnosis. Furthermore, basal cells can be patchy or discontinuous in some benign lesions. Consequently, negative staining for high molecular weight cytokeratin in a few atypical glands may not be sufficient for a definitive diagnosis of malignancy.[16] Therefore, there is the need for a positive marker for prostate cancer, and P504S is a good candidate for this problem.

The majority of diagnostic problems on prostate needle biopsies are related to small infiltrating malignant glands that are usually graded as Gleason score 3 + 3 = 6. In this study most of the cases (>95%) with a minute focus of small infiltrating glands of cancer had a Gleason score 3 + 3 = 6. Our results indicate that P504S has potential diagnostic value in assisting the pathologist to confirm the diagnosis of limited prostatic adenocarcinoma with Gleason score 3 + 3 = 6. High-grade (Gleason score >7) prostate cancer with extensive involvement of the prostate can be easily recognized on needle biopsy tissue by routine hematoxylin and eosin staining and rarely needs an adjunct study. However, small foci of high-grade prostate cancer can be occasionally seen on needle biopsy. In our study there were three cases of Gleason score >7. One of the cases with Gleason score 8 was negative for P504S. Therefore, the diagnostic value of P504S staining for detecting small foci of high-grade prostate carcinoma needs further investigation.

Although our findings suggest that P504S is an excellent marker for prostate adenocarcinoma, caution should be exercised in interpreting P504S immunohistochemical results for several reasons. First, P504S immunostaining of P504S was found in 69 of 73 cases and therefore is not 100% sensitive for prostate cancer. Negative P504S staining in small suspicious glands does not necessarily render a benign diagnosis. Although it is uncommon, focal nonreactivity of P504S has been previously reported in prostate cancer in prostatectomy specimens.[11] Routine hematoxylin and eosin staining with a combination of P504S and basal cell staining may be necessary in selected cases. The one negative P504S case in this study was foamy gland cancer. Whether a specific histologic subtype of prostatic adenocarcinoma, such as mucinous, foamy gland, signet-ring cell, or small cell carcinoma, can react with the P504S antibody remains to be studied.

Second, background P504S staining in smooth muscle and weak granular staining in benign glands have previously been reported[11] and could lead to false-positive results. However, in our hands this pattern of staining can be readily distinguished from dark and circumferential positive staining pattern of malignant glands. Moreover, in the present study immunostaining of P504S was highly specific for prostate cancer, and false positivity in benign glands was not a problem. A strong P504S reactivity was not seen in any of benign biopsies. Particularly benign glands adjacent to malignant glands provide excellent internal controls.

Finally, two possible premalignant lesions, high-grade prostatic intraepithelial neoplasia[11] and atypical adenomatous hyperplasia,[18] may exhibit some reactivity for P504S to a lesser degree based on our recent studies. Because both prostatic intraepithelial neoplasia and atypical adenomatous hyperplasia retain basal cells and are positive for 34βE12 immunostaining, which can be distinguished from prostate cancer when P504S immunoreactivity is present. Therefore, we recommend using a combination of P504S and 34βE12 when necessary to increase diagnostic accuracy.

In this study we excluded any case with small atypical glands suspicious but insufficient for diagnosis of prostate cancer. We think that it is important to confirm P504S expression in cases with definite evidence of carcinoma. Further studies of atypical glands with P504S immunostaining and clinical follow-up are necessary.

In summary, P504S immunohistochemistry detects minimal prostatic adenocarcinoma with high specificity and sensitivity on needle biopsy. Using P504S as a positive marker along with basal cell-specific 34βE12 as a negative marker could help to confirm the diagnosis of limited prostate cancer and reduce the chance of misdiagnosis on prostate needle biopsy.

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Exhibit E

Advances in Brief

Sequence Variants of α-Methylacyl-CoA Racemase Are Associated with Prostate Cancer Risk[1]

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Abstract

The enzyme α-methylacyl-CoA racemase (*AMACR*) plays an important role in peroxisomal β-oxidation of branched-chain fatty acid and therefore is relevant to carcinogenesis. The involvement of *AMACR* in prostate cancer (CaP) is implicated by the recent observation that expression of *AMACR* is consistently and extensively up-regulated in CaP. This observation is of particular interest, given previous findings from epidemiological studies that red meat and dairy products, major sources of branched-chain fatty acid, are associated with CaP risk and from linkage studies that the *AMACR* gene region at 5p13 is linked to a CaP susceptibility gene. In this study, we hypothesize that sequence variants in *AMACR* may alter the risk for CaP. To test this hypothesis, we sequenced all five exons, exon-intron junctions, the promoter region, and 3′-untranslated region of *AMACR* in germ-line DNA samples of 96 probands from hereditary CaP (HPC) families. Seventeen sequence variants, including five novel (R118Q, V185A, P238S, Q239H, and L250R) and five known (M9V, S52P, D175G, S201L, and K277E) missense changes, were identified. Six of these variants are at conserved residues among the rat and mouse *AMACR*. Eleven of these single nucleotide polymorphisms were genotyped in a total of 159 HPC probands, 245 sporadic cases, and 211 unaffected controls to assess their association with CaP risk. Significantly different genotype frequencies between HPC probands and unaffected controls were found for several missense changes, including M9V ($P = 0.03$), G1175D ($P = 0.02$), S291L ($P = 0.02$), and K277E ($P = 0.02$). Haplotype analysis provided stronger evidence for association ($P = 0.001$). Furthermore, the *AMACR* sequence variants strongly cosegregate with CaP in HPC families (log of odds = 3.78; $P = 0.00006$), especially in the subset of families whose probands carry the "A-A" haplotype of M9V and D175G (log of odds = 4.34; $P = 0.000008$). These results suggest that sequence variants in *AMACR* may be associated with CaP risk.

Introduction

AMACR[3] is a well-characterized enzyme that plays a key role in peroxisomal β-oxidation of dietary branched-chain fatty acids and C27-bile acid intermediates (1). It catalyzes the conversion of (*R*)-α-methyl-branched-chain fatty acyl-CoA esters to their (*S*)-stereoisomers. Only the (*S*)-stereoisomers can serve as substrates for branched-chain acyl-CoA oxidase during their subsequent peroxisomal β-oxidation. Recently, *AMACR* was found to be consistently and extensively overexpressed in CaP from multiple high-throughput gene expression profiling studies (2–5). Interestingly, epidemiological studies demonstrate that red meat and dairy products, which are both major sources of branched-chain fatty acid, are associated with CaP risk (6, 7). Furthermore, results from genome-wide scans for linkage in HPC families implicate 5p13, the chromosomal region of *AMACR*, as the location of a CaP susceptibility gene (8–10). Whereas the overexpression of *AMACR* is an important new marker of CaP, it is also possible that *AMACR* could play an important role in the etiology of CaP. We therefore hypothesize that sequence variants of *AMACR* may alter the risk for CaP.

To test this hypothesis, we sequenced all five exons, exon-intron junctions, the promoter region, and 3′-UTR of *AMACR* in germ-line DNA samples of 96 probands from HPC families to identify sequence variants. We genotyped a subset of sequence variants in a total of 159 HPC probands, 245 sporadic cases, and 211 unaffected controls to assess their association with CaP risk. Finally, we genotyped three sequence variants in 159 HPC families to assess the cosegregation and association between the sequence variants and CaP.

Materials and Methods

Subjects. A detailed description of the study sample was presented elsewhere (11). Briefly, 159 HPC families were collected and studied at the Brady Urology Institute at Johns Hopkins Hospital (Baltimore, MD). The diagnosis of CaP was verified by medical records for each affected male studied. Age of diagnosis of CaP was confirmed either through medical records or from two other independent sources. The mean age at diagnosis was 61 years. Eighty-four percent of the families were Caucasian, 6.9% were Ashkenazi Jewish, and 8.8% were African American. The average number of affected men per family was 5.08.

Two hundred and forty-five sporadic CaP cases were recruited from patients who underwent treatment for CaP at the Johns Hopkins Hospital and did not have affected first-degree relatives. The diagnosis of CaP for all these subjects was confirmed by pathology reports. Preoperative prostate-specific antigen levels, Gleason score, and pathological stages were available for 202, 240, and 241 cases, respectively. Mean age at diagnosis for these cases was 58.7 years, but family history information was not obtained. Over 93% of the cases are Caucasian, and 3.2% are African American.

Two hundred and twenty-two non-CaP controls were selected from men participating in screening programs for CaP. By applying the exclusion criteria of abnormal digital rectal examination and abnormal prostate-specific antigen level (*i.e.*, >4 ng/ml), 211 were eligible for the study. The mean age at examination was 58 years. More than 86% of the eligible controls are Caucasian, and 7.1% are African American. Based on interview with the controls, about 5.6% of the eligible controls have brothers or a father affected with CaP.

All individuals who participated in this study gave full informed consent.

Genotyping Methods. The PCR products of all five exons, exon-intron junctions, the promoter region, and 3′-UTR of *AMACR* were directly sequenced in 96 HPC probands. The primers for PCR are available upon request. All PCR reactions were performed in a 10-μl volume consisting of 30 ng of genomic DNA, 0.2 μM each primer, 0.2 mM each dNTP, 1.5 mM $MgCl_2$, 20 mM Tris-HCl, 50 mM KCl, and 0.5 unit of Taq polymerase (Life Technologies, Inc.). PCR cycling conditions were as follows: 94°C for 4 min; followed by 30 cycles of 94°C for 30 s, specified annealing temperature for 30 s, and 72°C for

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[3] The abbreviations used are: AMACR, α-methylacyl-CoA racemase; CaP, prostate cancer; UTR, untranslated region; HPC, hereditary prostate cancer; SNP, single nucleotide polymorphism; dNTP, deoxynucleoside triphosphate; HWE, Hardy-Weinberg equilibrium; LD, linkage disequilibrium; df, degrees of freedom; PIN, prostate intraepithelial neoplasia; LOD, log of odds.

Table 1 *Sequence variants of AMACR discovered in 96 Caucasian prostate cancer probands*

Position (bp)[a]	Location	Nucleotide change	Amino acid change	Allele	Allele frequencies (no. of alleles/no. of chromosomes) Caucasians		African Americans	
−90	Promoter	C/T		T	47/166	0.283	1/8	0.500
−70	Promoter	G/A		A	1/166	0.006	0/8	
25	Exon 1	A/G	M9V	G	72/166	0.434	5/8	0.625
154	Exon 1	T/C	S52P	C	1/168	0.006	0/8	
2,228	Exon 2	G/A	E118Q	A	0/172		2/10	0.200
3,236	Intron 2	T/A		A	0/174		1/10	0.100
3,420	Exon 3	A/G	D175G	G	73/172	0.424	7/10	0.700
9,197	Exon 4	T/C	V185A	C	1/180	0.005	0/10	
9,248	Exon 4	C/T	S201L	T	53/180	0.300	2/10	0.200
9,355	Exon 4	C/T	P238S	T	0/180		1/10	0.100
9,360	Exon 4	G/T	Q239H	T	15/180	0.083	1/10	0.100
18,557	Exon 5	T/G	L250R	G	1/172	0.006	0/10	
18,631	Exon 5	A/G	K277E	G	46/172	0.267	2/10	0.200
19,178	3′-UTR	T/G		G	0/174		3/10	0.300
19,568	3′-UTR	G/T		T	4/172	0.023	1/10	0.100
19,614	3′-UTR	G/A		A	59/174	0.339	1/10	0.100
19,665	3′-UTR	T/C		C	1/174	0.006	0/10	

[a] The position (bp) is based on the initiation codon (ATG) of *AMACR* genomic DNA.

30 s; with a final extension of 72°C for 6 min. All PCR products were purified using the QuickStep PCR purification Kit (Edge BioSystems, Gaithersburg, MD) to remove dNTPs and excess primers. All sequencing reactions were performed using dye terminator chemistry (BigDye; ABI, Foster City, CA) and then precipitated using 63 ± 5% ethanol. Samples were loaded onto an ABI 3700 DNA Analyzer after adding 8 μl of formamide. SNPs were identified using Sequencer software version 4.0.5 (Gene Codes Corp.).

SNPs were genotyped using the MassARRAY system (SEQUENOM, Inc., San Diego, CA). PCR reactions were performed in a total volume of 5 μl with 10 ng of genomic DNA, 2.5 mM $MgCl_2$, 0.1 unit of HotStarTaq polymerase (Qiagen, Valencia, CA), 200 μM dNTP, and 200 nM primers. The PCR reactions started at 95°C for 15 min, followed by 45 cycles of 95°C for 20 s, 50°C for 30 s, and 72°C for 1 min, with final extension of 72°C for 3 min. The extension reactions were performed in a total volume of 9 μl with 50 μM dNTP/dideoxynucleotide phosphate (ddNTP) each, 0.063 unit/μl Thermo Sequenase (both from SEQUENOM Inc.), and 600 nM extension primers. The cycling conditions were 94°C for 2 min followed by 55 cycles of 94°C for 5 s, 52°C for 5 s, and 72°C for 5 s. After cleaning up the extension reaction products with SpectroCLEAN, the products were transferred to SpectroCHIP using SpectroPOINT and then scanned through SpectroREADER. Genotyping was done using SpectroTYPER.

Statistical Methods. HWE test for each sequence variant and pairwise LD among all sequence variants were performed using the Fisher probability test statistic, as described by Weir (12). For each test, 10,000 permutations were performed, and the test statistic of each replicate was calculated. Empirical *P*s for each test were estimated as the proportion of replicates that is as probable or less probable than the observed data, as implemented in the software package Genetic Data Analysis (GDA).

Tests for association between sequence variants and CaP were performed by comparing the genotype frequencies between cases and controls using the χ^2 test with two df.

Haplotype frequency of unrelated individuals was estimated using the new statistical method proposed by Stephens *et al.* (13), as implemented in the computer program PHASE.big.[4] Several runs using different values for the seed of the random number generator were performed, and the goodness of fit values were similar among the different runs. Association between the haplotypes and CaP risk was performed using a score test developed by Schaid *et al.* (14), as implemented in the computer program HAPLO.SCORE.[5]

Linkage analysis was performed using the nonparametric method as implemented in GENEHUNTER-PLUS.[6] Allele sharing LOD values were calculated assuming an equal weight to all families using the computer program ASM (15). *P*s of the LOD were estimated by the formula $0.5*(1-(1-p_1)(1-p_1))$, where p_1 is the *P* value of χ^2 (4.6 × LOD) with one df.

A family-based association test was implemented using the computer program TRANSMIT (16).[7]

Results

Sequence analysis of all five exons, exon-intron junctions, the promoter region, and 3′-UTR of *AMACR* in the germ-line DNA samples of 96 probands from HPC families (91 were Caucasians and 5 were African Americans) revealed 17 sequence variants (Table 1). Among these sequence variants, 10 were missense changes. Five of these missense changes (M9V, S52P, D175G, S201L, and K277E) were reported previously (1). The remaining five missense changes (R118Q, V185A, P238S, Q239H, and L250R) were novel. Six of these missense changes (S52P, D175G, V185A, P238S, Q239H, and L250R) were at the residues that are conserved among the rat and mouse *AMACR*.

To assess whether these sequence variants are associated with CaP risk, 11 of these SNPs were genotyped in a total of 159 HPC probands, 245 sporadic cases, and 211 unaffected controls. These SNPs were chosen because they were observed more than one time in the Caucasian subjects of the 96 HPC probands and were either in the coding region or in the UTR. Because >90% of these subjects are Caucasians, all of the following analyses were limited to the Caucasians only to decrease the impact of potential population stratification. The frequencies of these SNPs in HPC probands (N = 133), sporadic cases (N = 229), and unaffected controls (N = 189) are listed in Table 2. Five of these SNPs were infrequent in these subjects; therefore, no formal statistical tests were performed due to insufficient power. The SNP in the 3′-UTR (19,614G/A) was significantly deviated from HWE in the controls (P = 0.038) and therefore was dropped from further analysis. The remaining five SNPs (M9V, D175G, S201L, Q239H, and K277E) were in HWE in the controls. These five SNPs were in strong LD because the tests for pairwise LD among these SNPs were all highly significant (all $P < 0.00001$). Significant differences in the genotype frequencies between HPC probands and unaffected controls were found for four of the five SNPs (M9V, D175G, S201L, and K277E). The alleles of "A" for M9V, "A" for D175G, "T" for S201L, and "G" for K277E were higher in HPC probands than controls. A similar, but not statistically significant, trend was found when the frequencies of these SNPs were compared between sporadic and unaffected controls. Considering the high degree of LD among these SNPs, haplotype analyses were used to further examine the association by analyzing these SNPs simulta-

[4] http://www.stats.ox.ac.uk/mathgen/software.html.
[5] http://www.mayo.edu/statgen for the S-PLUS programming language
[6] ftp://galton.uchicago.edu/pub/kong.
[7] http://www-gene.cimr.cam.ac.uk/clayton/software/.

Table 2 *Genotype frequencies of AMACR sequence variants in Caucasians*

SNP	Genotypes	No. of subjects (%)			χ^2 test for difference (*P*[a])	
		Controls	Sporadic	HPC probands	Sporadic vs. controls	HPC probands vs. controls
Frequent SNPs						
M9V	AA	53 (29.1)	74 (32.7)	39 (31.2)		
	GA	79 (43.4)	103 (45.6)	67 (53.6)		
	GG	50 (27.5)	49 (21.7)	19 (15.2)	0.38	0.03
D175G	AA	56 (29.8)	76 (33.8)	41 (31.1)		
	GA	82 (43.6)	100 (43.9)	72 (54.5)		
	GG	50 (26.6)	52 (22.8)	19 (14.4)	0.60	0.02
S201L	CC	93 (52.5)	128 (56.4)	62 (47.0)		
	CT	67 (37.8)	81 (35.7)	66 (50.0)		
	TT	17 (9.6)	18 (7.9)	4 (3.0)	0.70	0.02
Q239H	GG	123 (70.4)	165 (74.3)	106 (80.3)		
	GT	50 (26.5)	51 (23.0)	25 (18.9)		
	TT	6 (3.2)	6 (2.7)	1 (0.8)	0.67	0.08
K277E	AA	103 (56.6)	137 (59.8)	67 (51.1)		
	AG	63 (34.2)	77 (33.6)	59 (45.0)		
	GG	18 (9.8)	15 (6.6)	5 (3.8)	0.45	0.04
Rare SNPs (no statistical test for difference was performed due to small numbers)						
S52P	TT	181	223	123		
	TC	3	1	1		
R118Q	GG	186	226	129		
	GA	1	0	0		
V185A	TT	177	226	131		
	TC	1	0	1		
19.568G/T	GG	171	209	120		
	GT	17	20	11		
19.665T/C	TT	176	229	128		
	TC	0	0	1		

[a] Degree of freedom 2.

neously. Whereas the five-SNP haplotype analysis only provided weak evidence for association ($P = 0.04$), a two-SNP haplotype analysis of M9V and D175G provided stronger evidence for association. A significant global score test using the HAPLO.SCORE was observed when the haplotype between HPC probands and unaffected controls was compared (global-stat = 12.99; df = 2; $P = 0.001$). Further examination of the haplotype using the PHASE computer program showed that the haplotype "A-A" of M9V and D175G was more frequent in HPC probands (56.7%) than in unaffected controls (51.5%). A similar, but not statistically significant, trend was observed when haplotype between sporadic cases (55.2%) and unaffected controls was compared (global-stat = 3.91; df = 2; $P = 0.14$).

Because the stronger association of *AMACR* sequence variants and CaP risk was observed in HPC probands, additional analyses were performed to further evaluate the association using genotype-stratified linkage analysis and family-based association test. The three SNPs (M9V, D175G, and S201L) were genotyped in all available family members of the 159 HPC families. Multipoint linkage analysis in the complete set of 159 HPC families provided strong evidence for cosegregation between the sequence variants and CaP in these families, with a peak nonparametric allele sharing LOD of 3.48 ($P = 0.00006$; Table 3). Interestingly, this cosegregation appeared primarily in the families whose probands carried the "A-A" haplotype of M9V and D175G. The peak allele sharing LOD was 4.34 ($P = 0.000008$) in 120 families whose probands had this haplotype. In contrast, there was no linkage in the remaining 39 families (allele sharing LOD = 0). These results suggested that the "A-A" haplotype cosegregates with CaP risk in these families. Family-based linkage and association tests using the computer program TRANSMIT were also performed. However, no significant results were observed, probably because of the loss of information in this analysis due to (*a*) higher frequencies of homozygous haplotype carriers in parents and (*b*) trimming of extended families to nuclear families.

Table 3 *Nonparametric linkage analysis using genotypes at M9V, D175G, and S201L*

Families	No. of families	LOD	*P*
All	159	3.48	0.00006
Stratified by ethnicity			
Caucasians	133	2.47	0.0007
African Americans	14	1.60	0.03
Stratified by age of onset (yrs)			
≤65	79	1.22	0.02
>65	80	2.38	0.0009
Stratified by no. of affected			
3	29	0.50	0.12
4	40	1.04	0.03
≥5	90	1.97	0.003
Stratified by haplotype (M9V and D175G)			
A-A	120	4.34	0.000008
Not A-A	39	0.00	0.5

Discussion

Our hypothesis that sequence variants of *AMACR* may alter the risk for CaP is based on a series of important findings from epidemiological, genetic linkage, and gene expression profiling studies (2–9). An association study of *AMACR* sequence variants and CaP risk is a natural extension and an important next step of these previous studies. Our identification of multiple missense changes and other sequence variants in the gene and our finding that several of these missense changes are associated with CaP risk not only strengthen the claim that overexpression of *AMACR* is an important molecular marker for CaP but also suggest that *AMACR* may play an important role in the etiology of CaP.

Systematic interrogation of transcriptionally altered genes by high-throughput gene expression profiling indicated that *AMACR* was consistently and extensively overexpressed in a subset of CaP (2–3). Analysis of mRNA levels of *AMACR* revealed an average up-regulation of ~9-fold in clinical CaP specimens compared with normal specimens (4). The up-regulation of *AMACR* in CaP was further confirmed at protein level by both Western blot and immunohistochemical analysis in two independent studies (4–5). Luo *et al.* (4) demonstrated that both prostate carcinomas and high-grade PIN consistently scored significantly higher than matched normal epithelium. Rubin *et al.* (5) also found significantly higher mean *AMACR* protein staining intensity in specimens from atrophic prostate, PIN, localized CaP, and metastatic CaP, compared with specimens from benign prostate. Whereas these studies clearly demonstrated that overex-

pression of *AMACR* is an important marker for CaP, the precise mechanism of this overexpression is unclear. However, several pieces of evidence indicate that the overexpression of *AMACR* may increase the risk for CaP through the role of *AMACR* in tumor initiation and/or cancer cell growth. First, overexpression of *AMACR* is observed in high-grade PIN, a presumed cancer precursor lesion, suggesting that the overexpression of *AMACR* may be involved in the initiation of CaP. Second, the main sources of branched-chain fatty acids in the human diet (milk, beef, and dairy products) have been implicated as dietary risk factors for CaP. This association could be due to a combination of overconsumption of dietary branched-chain fatty acids that provides excess of substrates for the subsequent peroxisomal β-oxidation and the up-regulation of *AMACR* in the cancer cells that have a consistently greater capacity to metabolize dietary branched-chain fatty acids. Because overconsumption of dietary branched-chain fatty acids presumably occurs before cancer initiation and development, the link between over expression of *AMACR* and CaP could be causative. Third, the chromosomal region 5p13 is one of the most consistently reported positive linkage regions for CaP susceptibility genes from genome-wide scans (8–10). If this linkage is partially due to the candidate gene *AMACR* in this region, men who inherit *AMACR* mutations or certain polymorphic variants are predisposed to CaP. The inherited mutations coupled with somatic up-regulation of *AMACR* in some cells may lead to cancer initiation and development, perhaps due to increased β-oxidation with concomitant oxidative damage to DNA (4) or other unknown mechanisms.

This study was designed to assess whether *AMACR* is involved in CaP development using genetic approaches. Ten missense changes and seven other sequence variants in *AMACR* were observed in our study. Six of these missense changes were at the residues that are conserved among the rat and mouse *AMACR*, indicating potential functional implications of these changes. Four missense changes (M9V, D175G, S201L, and K277E) had significantly different genotype frequencies between HPC probands and unaffected controls, suggesting that these missense changes could be associated with CaP risk. The strong evidence for cosegregation between these SNPs and CaP risk in HPC families, especially in the families whose probands carry the "A-A" haplotype of M9V and D175G, provided additional support that these missense changes may be associated with CaP risk.

However, caution should be taken when interpreting our findings. Whereas the significant differences in genotype and haplotype frequencies in HPC probands and controls could be due to differential risks for CaP caused by these polymorphisms, they could also be due to other reasons, such as a type I error or population stratification. All of the reported significance levels were nominal *P*s and were not adjusted for multiple tests. If we considered that multiple tests were performed in this report (for CaP risk), and we used the commonly suggested Bonferroni correction, then none of the single SNP association tests would be significant. However, the Bonferroni correction is not optimal in this case because not all of these tests were independent, due to LD between these polymorphisms and the dependence between allele, genotype, and haplotype. As a case-control study, the results are subject to potential population stratification; that is, the different genotype frequencies observed may partially reflect different genetic backgrounds in cases and controls, especially considering the difference in the ascertainment schemes for HPC probands and unaffected controls. We are aware of this limitation and have tried to minimize the impact of the potential population stratification by performing the statistical tests in Caucasian subjects only. However, we feel that the significant difference in genotype and haplotype frequencies observed in this study is unlikely to be primarily due to the potential population stratification alone. The strong cosegregation of the risk haplotype and CaP risk in HPC families independently supports the idea that the sequence variants cosegregate and are associated with CaP risk.

It is surprising that the sequence variants and haplotype "A-A" of M9V and D175G imposed only a small risk to sporadic CaP, even though it segregated well with CaP in HPC families (suggesting a high penetrance) and was frequent in our population. The exact reason for this observation is unknown. However, one possible interpretation is that other major genes that interact with *AMACR* are frequent in HPC families but rare in the general population. It is also possible that variable timing and quantity of exposure to dietary branched-chain fatty acids, in the presence or absence of specific *AMACR* variants, could explain our interesting findings.

The findings of the association between sequence variants of *AMACR* and CaP risk in our study, if confirmed in independent studies and supported by functional studies, may have significant impact on our understanding of etiology, prevention, and treatment of CaP. The potential interaction between the overconsumption of dietary branched-chain fatty acids and sequence variants of *AMACR* needs to be further explored to understand the contribution of dietary risk factors and genetic factors and to ultimately develop effective clinical interventions to complement existing modalities.

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